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RECHTSVERGLEICHENDE DARSTELLUNG DER VATERSCHAFTSANGELEGENHEITEN IN DER SFR JUGOSLAWIEN MIT BESONDERER RÜCKSICHT AUF DIE REFORMBESTREBUNGEN IN DER SR MONTENEGRO

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EINLEITUNG

Als 1971 die Verfassungszusätze zur Verfassung der SFR Jugoslawien erlassen wurden, kam es zu Änderungen in der Verfassung aus dem Jahre 1963, wodurch sich in Jugoslawien das Verhältnis des Bundes zu seinen sechs Republiken und zwei autonomen Provinzen grundlegend neu gestaltete. Die gesetzgeberische Zuständigkeit der Republiken und der autonomen Provinzen erweiterte sich in dem Sinne, dass jede Republik und jede autonome Provinz das Recht erlangte, die familienrechtlichen Beziehungen auf ihrem Gebiet selbständig zu regeln. Eine solche teilweise Verlagerung der gesetzgeberischen Zuständigkeit nach unten übernahm auch die jetzige Verfassung der SFR Jugoslawien aus dem Jahre 1974. So erhielten alle Republiken und beide autonomen Provinzen auch die originäre Zuständigkeit zur rechtlichen Regelung von Vaterschafts-und Mutterschaftsangelegenheiten.

Alle Republiken und beide autonomen Provinzen haben die Vaterschafts-und Mutterschaftsangelegenheiten in ihre Familiengesetzgebung aufgenommen: in der SR Slowenien,[1] SR Kroatien,[2] SR Serbien[3] und in der SAP Kosovo[4] sind dies die Gesetze über die Ehe und Familienbeziehungen, in der SR Bosnien und Herzegowina[5] das Familiengesetz und in der SR Montenegro[6] und SR Mazedonien[7] sowie in der SAP Vojvodina[8] die Gesetze über die Beziehungen zwischen Eltern und Kindern.

1 Gesetz über die Ehe und Familienbeziehungen der SR Slowenien, Uradni list SRS, Nr. 15 vom 4.7.1976 (fortan: Sl-EheFamG).
2 Gesetz über die Ehe und Familienbeziehungen der SR Kroatien, Narodne novine SRH, Nr. 11 vom 21.3.1978 (fortan:Kroat-EheFamG).
3 Gesetz über die Ehe und Familienbeziehungen der SR Serbien, Službeni glasnik SRS, Nr. 22 vom 7.6.1980 (fortan:Sb-EheFamG).
4 Gesetz über die Ehe und Familienbeziehungen der SAP Kosovo, Službeni list SAPK, Nr. 10 vom 28.3.1984 (fortan: Kos-EheFamG).
5 Familiengesetz der SR Bosnien und Herzegowina, Službeni list SR BiH, Nr. 21 vom 9.6.1979 (fortan: B-H FamG).
6 Gesetz über die Beziehungen zwischen Eltern und Kindern SR Montenegro, Službeni list SR CG, Nr. 54 vom 31.12.1975 (fortan: Mo-BezElKG).
7 Gesetz über die Beziehungen zwischen Eltern und Kindern der SR Mazedonien, Službeni vesnik na SRM, Nr. 5 vom 19.2.1973 (fortan: Maz-BezElKG).
8 Gesetz über die Beziehungen zwischen Eltern und Kindern der SAP Vojvodina, Službeni list SAPV, Nr. 2 vom 20.2.1975 (fortan: Voj-BezElKG).

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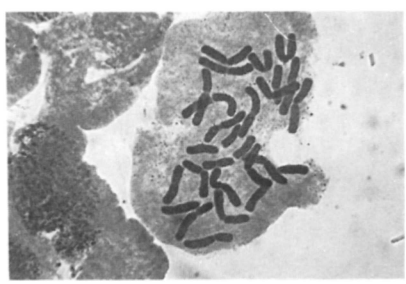

BILD 1.: Unbeschädigte - normale
Fichtenchromosomen

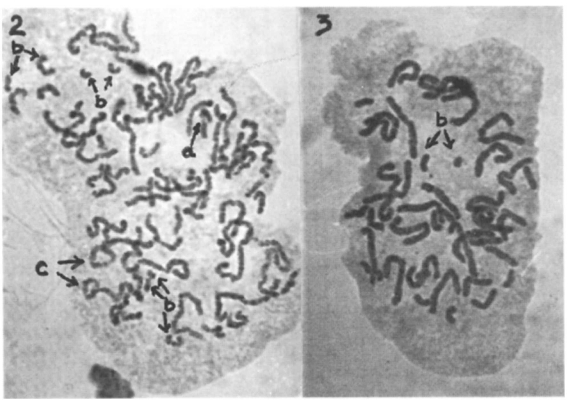

BILD 2 und 3: Einige specifische Aberrationen:
a. Lücke , b. Fragment, c. Ring

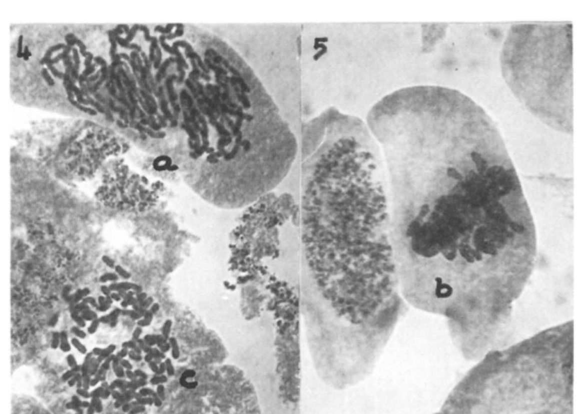

BILD 4 und 5: Einige unspecifische
Aberrationen:
a. Verbindungen,
b. Verklebungen
c. Centromermißteilung

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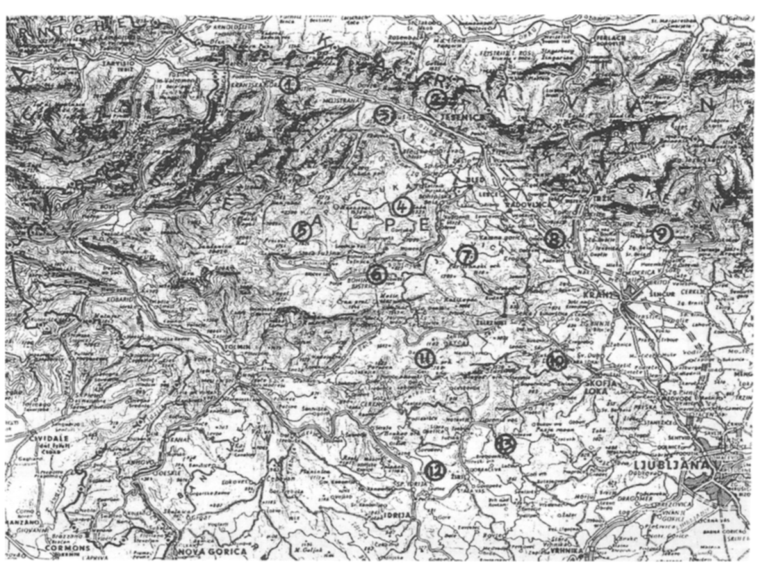
AI, die Summe verschiedener Typen der sogenannten spezifischen (Bild 2 und 3) und unspezifischen (Bild 4 und 5) Abarrationen, ergab 90 %, was ungefähr 3.5 mal mehr ist, als die bis jetzt festgestellte niedrigste Frequenz (Kontrole) der Beschädigung des genetischen Stoffes der Fichte in Slowenien. Es ist auch der höchste Prozent von spezifischen Aberrationen (besonders der Fragmenten und Ringe), was vor allem ein Nachweis der radioaktiven Bestrahlung ist.

TABELE 1.: Übersicht der Belastung des genetischen Materials der Fichte auf verschiedenen Lokalitäten im Oberkrain (Slowenien, Jugoslawien)

Lokalität	% der Beschädigung des gen. Materials (AI)	% der charakteristischen specifischen Aberrationen	% der charakteristischen unspecifisch.Aberrationen	Beschädigungsklasse	Vergrösserung AI in Vergleichung mit der Kontrole	N
Kontrole	26.50	2.17	12.74	1		500
1. Martuljek	59.72	-	29.37	+3	2.25x	378
2. Planina p.Golico	68.67	11.00	35.00	4	2.59x	300
3. Mežakla	56.00	2.00	35.00	+3	2.11x	200
4. Pokljuka	40.00	2.00	24.00	+2	1.51x	300
5. Fužinske planine	43.48	6.52	13.04	-3	1.64x	46
6. Bohinjska Bistr.	49.44	2.24	24.96	3	1.86x	625
7. Jelovica	37.00	-	30.00	+2	1.40x	300
8. Ljubno na Gor.	41.44	2.67	20.00	-3	1.67x	300
9. Javorov vrh	48.33	7.67	19.34	3	1.82x	300
10. Lubnik	30.33	9.33	7.00	-2	1.15x	300
11. Davča	50.36	2.50	20.72	3	1.90x	280
12. Žiri	47.11	6.00	18.67	3	1.78x	450
13. Žirovski vrh	90.48	23.19	17.49	+4	3.41x	263

Klasse	% des beschäd.gen.Mat.
1	- 30
2	31 - 40
3	41 - 60
4	61 -



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- Wir haben eine Abweichung von der slowenischen Population auch bei den MN Antigenen festgestellt.

Schlussfolgerung:

Anomalien der Rh Chromosome haben wir bei 10 Personen, die aus Žirovski vrh entstammen festgestellt. Bei dennen haben wir 3 mal die Mutter ausgeschloßen, 2 mal den Vater.

Zwei Familien, dennen die ausgeschlossenen Mütter angehören sind miteinander verwandt, die dritte nicht.

Die beiden ausgeschlossenen Väter sind miteinander nicht verwandt.

Die Frau II. - 3 ist Tochter von IV. - 7, was die Erblichkeit des anormalen Chromosoms in der 3. Generation beweist.

Alle Ergebnise werden bestätigt:

Dr.C.M.Giles - WHO, London 1979 Int.Blood Group Ref.Lab.; Dr.Culliford, Dr.Martin - The Metropolitan Police Forensic Science Lab., London 1979; Dr.P.Tippett - Medical Research Council - Blood Group Unit University College London 1984.

Die Fortsetzung der Studie folgt.

B. Druškovič

Auf dem Gebiete Žirovski vrh haben wir auch cytogenetische Analysen (die Feststellung der Typen und der Frequenzen des beschädigten genetischen Materials) an den Wurzelspitzen verschiedener Pflanzenarten durchgeführt. So haben wir bei der Fichte (Picea abies/L./Karsten) zum Beispiel der höchste Prozent des beschädigten genetischen Materials sowohl im Oberkrain (Tabele 1) wie auch im ganzen Gebiete Sloweniens gefunden. Der Aberrationsindex -

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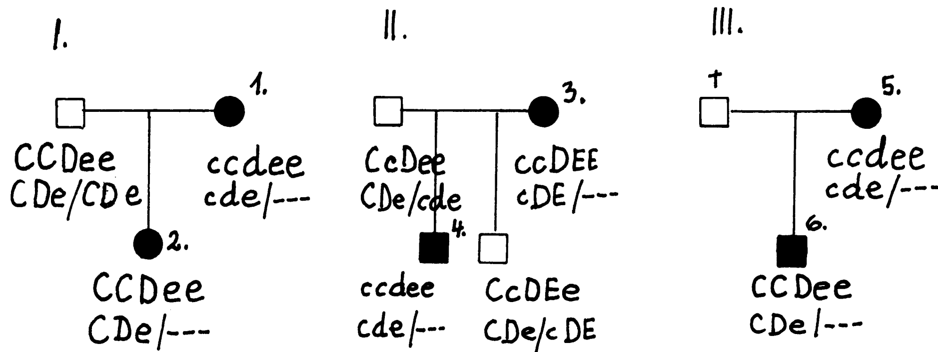
Blutgruppen untersucht. Testiert haben wir auch die übrigen Blutsysteme.

Verwendete Sera: Biotest, Immunno, Ortho, unsere eigene

Effektdosen: Autoanalisator Technicon

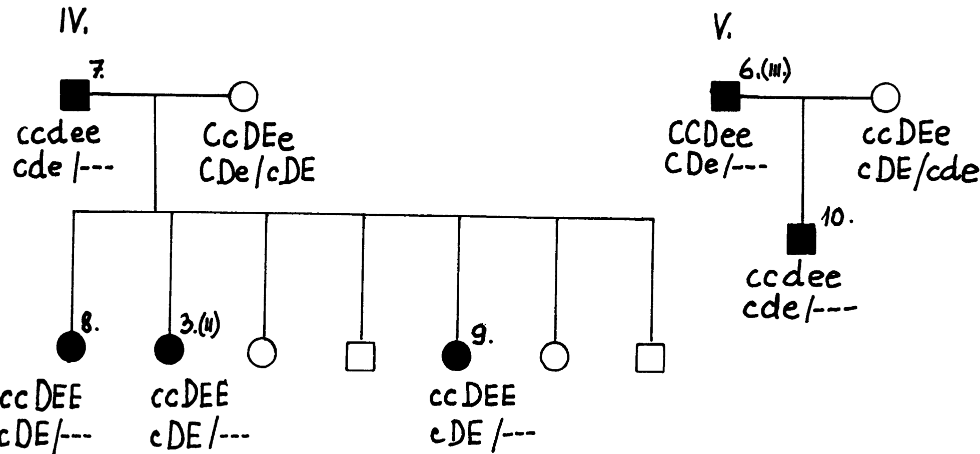
Ergebnisse: Ausschließen der Mutter in 3 Fällen



- Die Mutter 1. und 2. gehören derselben Familie an, sind also in senkrechter Folge in Verwandtschaft.

Die Mutter Nr.3 ist aus einer ganz anderen Familie.

- Die Anzahl der Delezionen, wo wir den Vater ausschließen:



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DIE BELASTUNG DES GENETISCHEN MATERIALS IM SLOWENISCHEN GEBIET ŽIROVSKI VRH (Uranium radioaktive Strahlung), prof. dr. Ljerka Glonar, Zavod SRS za transfuzijo krvi, Ljubljana, Yugoslavia, Dr.B.Druškovič, Institut za biologijo Univerze Ljubljana, Yugoslavia.

L. Glonar

Im Bereiche des Uranerzlagers Žirovski vrh ist seit einigen Jahren ein Bergwerk. Anläßlich Gelegenheitserforschungen einer Rh sensibilisierten Frau haben wir entdeckt, daß wir sie unter Beachtung der Rh Blut-Gruppe, als Mutter ihrer eigenen Tochter ausscheiden müssen. Auch haben wir testiert das Blut des Bruders dieser Frau und das Blut seiner Familienmitglieder. Die Ergebnisse, die eine Abweichung von der Normale zeigen, haben wir mehrmals wiederholt und auch in Beglaubigung entsendet. Von diesem Studienstück haben wir auch schon berichtet.

Weil es für einen Ausnahmsfall geht, haben wir uns entschieden, dass wir mehrere Familien, die in diesem Gebiet leben, untersuchen werden.

Anzahl der untersuchten Personen: 224; Familien: 11.

Material und Arbeitsmetoden

1. Besuche der Familien in ihrem Heim

   Wir haben die Orte besucht, wo die grösste Strahlung festgestellt wurde (M.Strle); Wir haben die Familien in ihren Häusern besucht, sowohl auch die wegziehenden Familienmitglieder; Die Bemerkungen der Einwohner: die Aenderungen der Farbe der Pflanzen, üppigeres Grün, schnelleres Wachstum.

2. Testieren der Blutmuster

   Hinsichtlich auf die gewonnene Resultate haben wir vor allem die Rh

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Other groups showed no significant frequency of blood groups and single malignant illnesses.

CONCLUSION

When comparing ABO blood groups and Rh D system between groups of patients treated for malignant tumors of different localizations and healthy persons,the following results were gained:

- low frequency of A blood group, associated with high frequency of O blood group, and high frequency of Rh (D) negativity, established in patients with breast malign tumour,were statistically significant.
- High frequency of Rh (D) negativity found in patients with gastrointestinal malignancy,was also statistically significant.
- High frequency of B blood group found in patients with sarcomas was not significant,
- In groups of patients with malignant hemopathies,melanoma malignum, respiratory system malignancies and gynaecologic malignancies were not found any statistically significant differences.

The analysis of blood groups of patients with narrower localization, like for example,only patients with stomach tumor, or colon tumor, would probable give different results and this exactly is the major task of our next searches. This problem **demands** further searches.

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Table 2. FREQUENCY OF Rh (D) BLOOD GROUP

MALIGNANT DISEASES		TESTED PATIENTS	Rh D BLOOD GROUP POSITIVE	NEGATIVE	RELATIVE INCIDENCE	χ^2 TEST +
BREAST	No.	2.984	2.451	533	o.8592	9.o9o6
	%	loo	82.14	17.86		
GASTROINTESTINAL	No.	2.957	2.436	521	o.8736	7.o78o
	%	loo	82.38	17.62		
RESPIRATORY SYSTEM	No.	2.445	2.o57	388	o.99o5	o.o274
	%	loo	84.13	15.87		
GYNAECOLOGY	No.	1.752	1.465	287	o.9537	o.5o86
	%	loo	83,62	16.38		
CENTRAL NERVOUS SYSTEM	No.	1.689	1.433	256	1.o458	o.4185
	%	loo	84.84	15.16		
SARCOMAS	No.	44o	363	77	o.88o8	1.o234
	%	loo	82.5o	17.5o		
MALIGNANT HEMOPATHIES	No.	389	318	71	o.8368	1.8158
	%	loo	82.5o	17.5o		
MELANOMA MALIGNUM	No.	24o	195	45	o.8o96	1.6163
	%	loo	81.25	18.75		
CONTROL GROUP	No.	29.983	25.263	4.72o		
	%	loo	84.25	15.74		

+ hi^2 test

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Table 1. FREQUENCY OF ABO BLOOD GROUPS

MALIGNANT DISEASES		TESTED PATIENTS	A	B	AB	O	RELATIVE INCIDENCE A/O	χ^2 TEST+	RELATIVE INCIDENCE B/O	χ^2 TEST+
BREAST	No.	2.984	1.172	524	218	1.070	0,8897	6.951	1.0059	0.0099
	%	100	39.28	17.56	7.30	35.86				
GASTROINTESTINAL	No.	2.957	1.255	498	206	998	1.0214	0.2297	1.0247	0.1828
	%	100	42,44	16.84	6.96	33.76				
RESPIRATORY SYSTEM	No.	2.445	1.000	411	198	836	0.9716	0.3495	1.010	0.0268
	%	100	40.90	16.81	8.10	34.19				
GYNAECOLOGY	No.	1.752	747	304	108	593	1.0231	0.1645	1.0527	0.5052
	%	100	42.64	17.35	6.16	33.85				
CENTRAL NERVOUS SYSTEM	No.	1.689	671	313	129	576	0.9462	0.8971	1.1158	2.3027
	"	100	39.73	18.53	7.64	34.10				
SARCOMAS	No.	440	178	91	27	144	1.004	0.014	1.2976	3.7421
	%	100	40.45	20.68	6.14	32.73				
MALIGNANT HEMOPATHIES	No,	389	154	59	30	146	0.8568	1.7807	0.8298	1.4754
	%	100	39.59	15.17	7.71	37.53				
MELANOMA MALIGNUM	No.	240	106	34	19	81	1.0629	0.1918	0.8442	0.7259
	%	100	44.17	14.17	7.91	33.75				
CONTROL GROUP	No.	29.983	12.582	4.977	2.204	10.220				
	%	100	41.96	16.60	7.35	34.09				

+ **hi^2 test**

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BLOOD GROUPS IN PATIENTS WITH MALIGNANT DISEASES

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INTRODUCTION

Many associations between blood group frequency and disease have been reported, most of the early studies can be criticized on technical and statistical grounds,and few have been confirmed. Numerous studies have confirmed that group A people have about a 2o percent greater chance of developing cancer of the stomach than group 0 people, while group 0 people have a 2o percent greater chance of developing duodenal ulcer than people of other ABO types.

PATIENTS AND METHODS

The study deals with ABO and Rh D blood system in all patients treated in the Central Institute for tumors in the course of 14 years (1st January 1971 - 31st December 1985.). During this period 12.896 patients divided due to tumor localization were treated. The majority were 2984 female patients treated for breast malignant tumor,most of whom had breast scirosum carcinoma. The second group of 2957 patients had adenocarcinoma localized in stomach and colon. The third group consisted of patients with planocelular carcinoma of cavi oris,respiratory system and esophagus. The fourth group of 1752 patients had malignant tumor of uterus and ovary.The fifth group of 1689 patients were treated for malignant tumor of central nervous system and medula spinalis. The sixth group of 44o patients had sarcoma. Hemoblastosis included patients with Morbus Hodgkin, non Hodgkin lymphoma, different types of leukemia and plasmocytomas. This group consisted of 389 patients. The last were 24o patients treated for malignant melanoma. Blood groups ABO and Rh D were determined in a routine search.The control group consisted of 29,983 healthy Yugoslav soldiers.

RESULTS

All the patients were compared with the healthy control group. Statistical evaluation showed greater frequency of patients with 0 blood group and Rh D negativity in the group of female patients with breast malignant tumor. The second group which consisted of 2957 patients with gastrointestinal tumors when compared with 29,983 healthy persons showed greater number of patients with Rh D negativity. **/ Table 1.and 2./**

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quintile with success chances between 60% and 80% and between 40% and 60% respectively and with the group systems: Rh, ADA and PGM_1, Bf, HP, MN respectively. The determination of these systems should be tried in any case. The chances of success are not excellent but sufficient (Sachs et al. 1986). The chances of success in the determination
of the group systems of the last two quintiles: EsD, P, GPT, Ch_1, Tf, Kell, Fy, Lewis, Xg, C3, GLO, Lu and Jk are so limited that the investigation should be tried only in special cases.

Summary

The success of determination of genetic blood markers in old stored hemolytic and alcohol containing blood samples depends lesser on the age of the samples but more on the susceptibility or resistance of the various group systems against autolysis, contamination pathogens etc. For this reason it is recommended first to determin group systems with a high chance of success like ABO, Gm, Km, 6-PGD, AK, Gc, acp, Rh, ADA and possibly still PGM_1 and Bf. Other group systems with limited chances of success should be determined only in special cases.

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The obviously closer association between the determination success and the different group systems can be demonstrated in a diagram with the group systems on the abscissa and the percentages of success on the oridinate (Fig.1). Subdividing the percentage scale in quintiles there are five blocks of group systems each with decreasing chances of determination success. The group systems: ABO, Gm, Km, 6-PGD, AK, Gc and acp are in the first quintile with success chances between 80% and 100%. Therefore it is reasonable to start an investigation with these seven systems. Next comes the second and third

Fig.1: Diagram of the mean percentage of success determining 26 blood group systems of stored blood samples.

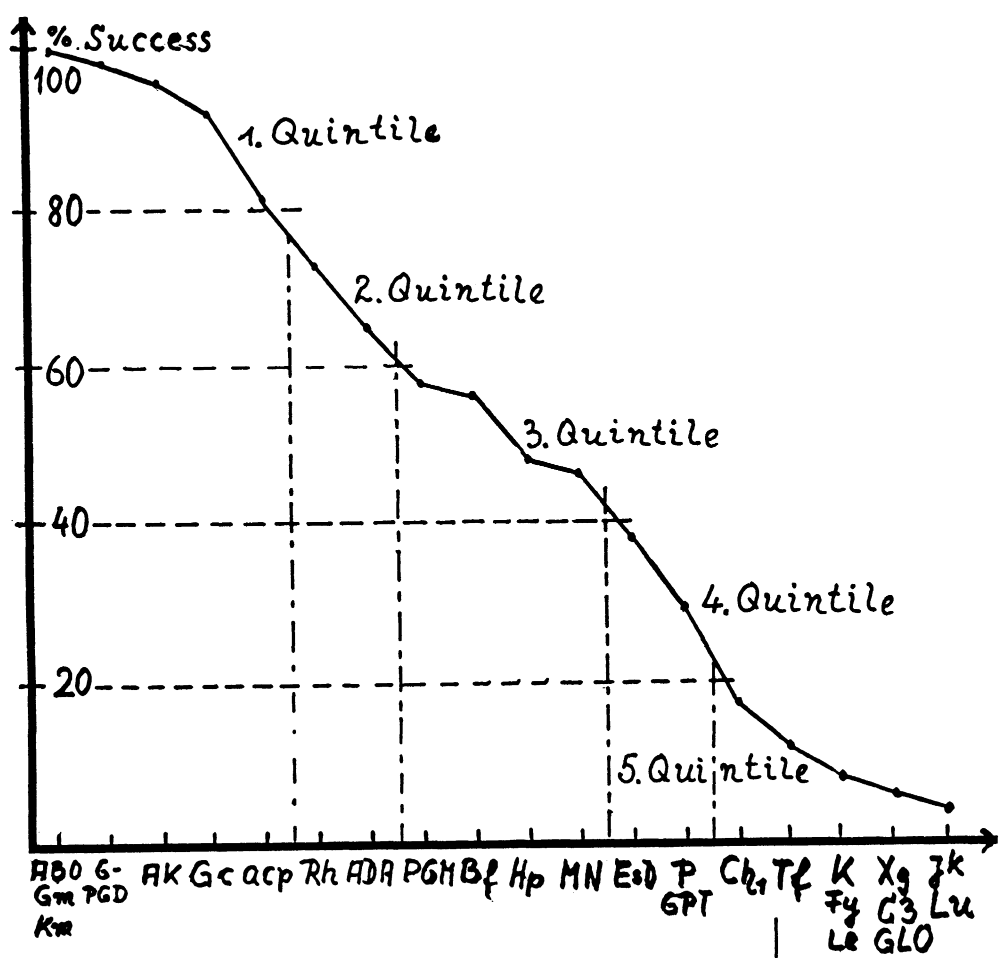

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Table 1b: Number of successful determinations of serum groups in 52 stored blood samples

Age of Sample Mounths	Serum Group Systems Hp	Gc	Gm	Km	Ch_1	C3	Bf	Tf	Total Number of Samples
1	1	1	1	1		1	1	1	1
2	1	3	3	3			2		3
3	1	5	5	5	1		2	1	5
4	6	8	11	11	2	1	9	1	11
5	7	12	13	13	2		5	1	13
6	1	8	8	8	1		3	1	8
7	2	2	2	2			2		2
8	3	4	4	4	3	1	2	1	4
9	1	2	2	2			1		2
10	1	1	1	1			1		1
11		1	1	1			1		1
12	1	1	1	1					1
Sum	25	48	52	52	9	3	29	6	52
% Success	48	92	100	100	17	6	56	12	100

Table 1c: Number of successful determination of erythocyte enzyme groups in 52 stored blood samples

Age of Sample Months	Erythrocyte Enzyme Group Systems acp	PGM_1	AK	ADA	6-PGD	EsD	GPT	GLO	Total Number of Samples
1	1	1	1	1	1	1		1	1
2	3	2	3	1	3	1			3
3	5	5	5	5	5		4		5
4	8	9	11	6	11	6	4		11
5	11	5	12	9	13	6	4	1	13
6	6	3	8	6	8	2	1		8
7	1	1	2	1	2			1	2
8	4	3	4	2	4	3	1		4
9	1		2	1	2		1		2
10	1	1	1	1	1				1
11			1	1	1	1			1
12	1								1
Sum	42	30	50	34	51	20	15	3	52
% Success	81	58	96	65	98	38	29	6	100

In Table 1 the percentages of success per system decreases successively from 100% to 4%. There are high middle and low determination successes. Surprisingly the influence of the sample's age seems to be minimal, except in very short and very long time stored samples.

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Blood Group determination in stored alcohol containing blood samples for identity examination.

V.Sachs, R.Doerner, B.Vollert

Once in a while blood group serologists have to determine genetic blood markers in older stored alcohol containing hemolytic and possibly contaminated blood samples for identity examination. In this matter the arising problems and especially the risk of false results have been discussed from OEPEN (1982), OSTERHAUS and BIRKNER (1982,1985) as well as STRATTON and RENTON (1958). In order to have an information in such cases whether the investigation of certain blood group systems is successful or not and about the possibly influencing factors we have analysed the results of 52 investigated samples.

The tables 1a, b and c show the number of successful determinations arranged to increasing sample ages and to blood group, serum group and erythrocyte enzyme group systems.

Table 1a: Number of successful determinations of blood groups in 52 stored blood samples

Age of Sample Months	Blood Group System Systems										Total Number of Samples
	ABO	MN	Rh	P	Kell	Fy	Jk	Le	LU	XG	
1	1	1	1	1	1	1	1	1		1	1
2	3	1	3								3
3	5	2	3	1						1	5
4	11	5	8	2							5
5	13	7	11	5	1	1		1	1		13
6	8	5	6	2		1		1			8
7	2										2
8	4	2	2	2	1	1	1	1	1	1	4
9	2	1	1	1	1						2
10	1		1								1
11	1		1	1							1
12	1		1								1
Sum	52	24	38	15	4	4	2	4	2	3	52
40 Success	100	46	73	29	8	8	4	8	4	6	100

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Table 1. Frequency of silent genes in 10 different systems, determined a) by estimates using the maximum-likelihood method, and b) on the basis of the frequencies of "incompatible" mother-child combinations.[1,2]

run.no.	system	f(*O) max.-likel.-estimates	no. of persons	chi^2	fg	$\underline{X}$ empir.determined	f(*O) freq.of mother-child	$\overline{X}$ "incomp."	N doub-lets	k m.-ch. "incomp."
1	acP(A;B;C)	0.0000	1.800	4.872	1	0.00020...	0.00060...	0.00160	11.700	4
2	Tf(C1;C2;C3;B;D)	0.0000	1.017	0.00		0.00400...	0.02200...	0.08190	2.200	1
3	Jk(a;b)	0.0005	6.297	0.00		0.00024...	0.00080...	0.00198	11.000	4
4	GLO(1;2)	0.0006	5.862	0.00		0.00080...	0.00160...	0.00300	12.177	10
5	Bf(F;F_1;S;SO,7)	0.0008	1.414	3.69	2	0.00030...	0.00090...	0.00220	13.217	4
6	Hp(1;2)	0.0014	7.315	0.01		0.00026...	0.00080...	0.00198	11.000	4
7	PLG(A;B;M;A_V;B_V)	0.0052	1.490	5.17	3	0.00130...	0.00390...	0.00990	2.200	4
8	Pi(M1;M2;M3;S;Z;V)	0.0065	3.454	15.80	11	0.00004...	0.00090...	0.00480	2.717	1
9	EsD(1;2;5)	0.0074	1.825	1.96	1	0.00037...	0.00138...	0.00400	11.600	3
10	PGM_1(1;2)	0.0100	4.756	0.48		0.00150...	0.00290...	0.00510	11.500	12

[1] In order of increasing frequency of the silent gene (determined by estimates using the maximum-likelihood method)

[2] excluded the systems ADA, AK, C3, C6, F13B, Gc and 6-PGD systems, for which neither estimates nor empirical survey produced indications of a silent gene, as well as the MNSs system which has so far defied methods of simple calculation

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quency of the silent gene of 0.0016, i.e. just above the limiting value of 0.00135. Nevertheless it seems not justified to include a silent gene in the GLO gene list.

In the systems Hp and Pi the M.-L.-estimates produced silent-gene frequencies of 0.0014 and 0.0065 respectively, i.e. above 0.00135. However, as the empirical survey does not support these values these systems do not have to be extended to include a silent gene either.

This leaves the systems PLG, EsD and PGM_1. In all three both estimates and empirical survey indicate a silent gene with a frequency of more than 0.00135. Hence, in new biostatistical tables these 3 systems should be expanded to include a silent gene, as was done some time ago in the cases of the systems Duffy, GPT and Kell-Cellano.

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$$p = \sum_{i=1}^{n} \sum_{\substack{j=1 \\ i \neq j}}^{n} a_i \cdot a_j \cdot a_o = a_o \sum_{i=1}^{n} a_i \left[\sum_{\substack{j=1 \\ j \neq i}}^{n} a_j + a_i - a_i \right]$$

$$\approx a_o \sum_{i=1}^{n} \left[a_i (1-a_i) \right],$$

where n is the number of non-silent genes. The number of "incompatible" mother-child doublets, K, to be expected among N examined doublets is $K = p \cdot N$.
The frequency of the silent gene on the basis of found "incompatible" mother-child doublets will then be

$$a_o = K/N \cdot 1 / \sum_{i=1}^{n} \left[a_i (1-a_i) \right] ,$$

where a_i $(i=1,\ldots,n)$ is the frequency of non-silent gene in the system.

So far, the existence of silent genes with a frequency greater than 0.00135 has been empirically established - i.e. using "incompatible" mother-child combinations - in the systems Duffy, GPT and Kell-Cellano. Current biostatistical tables take this into account (Hummel 1986). Accordingly, in these systems opposite homozygosity between child and pf no longer results in exclusion of the man from paternity; instead, the result is a high EM value. - One can also estimate the frequency of silent genes using the maximum-likelihood technique. If, using at least 3 phenotype frequencies, one determines not n but n+1 gene frequencies, then, as a rule, the Hardy-Weinberg law of panmixia will be better fulfilled if a silent gene is present than if not. -
Table 1 (see below) compares the results of an empirical survey on silent gene frequencies in Germany[1)] with those obtained from statistical estimates.

As the table shows, there are, for the most part, only minor differences between the empirically determined frequencies of silent genes and the estimates by maximum likelihood. -
Neither the estimates nor the empirical survey produced any indication of the existence of a silent gene of determinable frequency in the systems ADA, AK, C3, C6, F13B, Gc or 6-PGD (not in the table). The existence of silent genes has been proven for the systems acP, Kidd and Bf; however, both the estimated and empirical frequencies are lower than 0.00135.

In the Tf system the single occurrence of a mother-child "incompatibility" in 2,200 doublets leads mathematically to a relatively high frequency of the silent gene. But this frequency is not supported by the maximum-likelihood estimates. Hence, one may assume that the silent gene frequency in the Tf system lies below 0.00135. - In the GLO system, too, the calculations with the phenotypes produced a low silent gene frequency; however, among 12,177 mother-child doublets 10 were incompatible, giving a fre-

[1]For the kind cooperation we are very indebted to the colleagues Prof.Berg, Dr.Fingscheidt, Prof.Goedde, Prof.Hilgermann, PD Dr. Höher, Prof.Jürgens, Dr.Luboldt, Dr.v.Pritzbuer, Prof.Pulverer, Dr.Röhrborn, Dr.Werner, Prof.Wuermeling, Prof.Zang

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On the question of the reliability of silent gene frequencies derived from maximum-likelihood estimates

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On account of the relative high frequency of homozygous people with blood group O or Rh-negative it does not cause any problems to determine frequencies of the silent genes O or d. But this is not the case in systems in which the silent gene is rare. In some cases proof of a man's paternity rests on both he and the child possessing a rare silent gene. In such cases the man will mostly be heterozygous with respect to this gene. In serostatistical terms this means that the triplet in question can give only a negative indication of paternity; moreover, the rarer the gene, the more negative the indication.

In contrast to variant genes, the biostatistical value of silent genes depends on the kind and frequency of the other alleles in the system. Given heterozygosis for the silent gene in child and putative father (pf) and a frequency of 0.01, one will obtain e.g.

in the Fy(a,b,O) system EM = 11.383, and
in the K(1,2,O) system EM = 10.580.

An EM limit for the inclusion or non-inclusion of a rare silent gene in a gene list presupposes that the other allele frequencies in the system are fixed. Alone the problems raised by aliens renders this impracticable. As a solution to the problem we propose the fiction of proof of the silent gene. In this event one would always obtain a positive indication. A frequency of 0.00135 for the silent gene would produce an indication of W = 99.73% and the corresponding predicate "practically proven". Rare silent genes could then be placed in the same category as rare variants: genes with frequencies below the proposed frequency limit of 0.00135 would not be included in gene lists for biostatistical tables, whereas those with frequencies above would (Hummel 1983).

The existence of a rare silent gene is shown by the occurrence of genetically "incompatible" mother-child combinations. The frequency of this gene can be calculated from the frequency of these combinations:

In a case of "incompatibility" let the genotype of the child be A_iO, that of the mother A_jO. Then $a_i \cdot a_j \cdot a_O$ will be the frequency of the "incompatible" mother-child doublet. The probability of such "incompatibilities" is

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$$H = \sum_{i=0}^{n} \sum_{j=0}^{m} x(i,j) \ln \left[x(i,j)^{(0)} / x(i,j) \right] \quad \text{since}$$

$$H = \sum_{i=0}^{n} \sum_{j=0}^{m} x(i,j) \ln \left[x(i,j)^{(0)} \alpha(i) \beta(j) / x(i,j) \right]$$

$$= \sum_{i=0}^{n} \sum_{j=0}^{m} x(i,j) \left\{ \ln \left| x(i,j)^{(0)} / x(i,j) \right| + \ln \alpha(i) + \ln \beta(j) \right\}$$

$$= \sum_{i=0}^{n} \sum_{j=0}^{m} x(i,j) \ln \left[x(i,j)^{(0)} / x(i,j) \right]$$

$$+ \sum_{i=0}^{n} \ln \alpha(i) \sum_{j=0}^{m} x(i,j) + \sum_{j=0}^{m} \ln \beta(j) \sum_{i=0}^{n} x(i,j)$$

$$= H + \sum_{i=0}^{n} a(i) \ln \alpha(i) + \sum_{j=0}^{m} b(j) \ln \beta(j) = H + \text{const.}$$

In conclusion it should be noted that

1. if some elements $x(i^*,j^*)$ of the matrix X are fixed from the beginning, the corresponding elements $x(i^*,j^*)^{(0)}$ in X_0 should be nullified and $x(i^*,j^*)$ will be substracted from $\bar{a}(i^*)$ and $\bar{b}(j^*)$. After the application of the algorithm, the nullified elements with indices (i^*,j^*) should be substituted for $x(i^*, j^*)$.
2. In HlA matrix alleles A(O), B(O) are collective terms for unprovable features. If not all of the $\bar{a}(i)$ and $\bar{b}(j)$ have been defined, then it is necessary to reduce the initial matrix X_0 to the corresponding size: if some $\bar{a}(i^*)$ $(\bar{b}(j^*))$ are not defined, the line i^* (row j^*) of the matrix X_0 will be nullified and its elements will be substracted from the elements of the line O (row O).

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P.S. We produced HLA-A,B haplotype frequency matrices for more than 72 europid sub-populations and for 164 non-europid populations.

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on $x(i,j)^{(0)}$ will be discussed below. A sum of any line i in this matrix will not necessarily be equal to $\bar{a}(i)$. At the next step the matrix will be transformed to obtain this equality in each line: every element $x(i,j)^{(0)}$ will be multiplied

with $\bar{a}(i)/\sum_{j=0}^{m} x(i,j)^{(0)}$

to produce $x(i,j)^{(1)} = x(i,j)^{(0)}\bar{a}(i)/\sum_{j=0}^{m} x(i,j)^{(0)}$,

elements of matrix $X_1 = \{x(i,j)^{(1)}\}$. A sum of any column j in X_1 will not necessarily be equal to $\bar{b}(j)$. At the next step X_1 will be transformed to obtain this equality in each column: every element $x(i,j)^{(1)}$ will be multiplied with

$$\bar{b}(j)/\sum_{i=0}^{n} x(i,j)^{(1)}$$

to produce $x(i,j)^{(2)} = x(i,j)^{(1)}\bar{b}(j)/\sum_{i=0}^{n} x(i,j)^{(1)}$,

elements of matrix $X_2 = \{x(i,j)^{(2)}\}$. In X_2 the lines again should be improved etc. The subsequent improvements of lines and columns as described above establish an infinite sequence of matrices X_k which, when $k \to \infty$, converges (Bregman 1967) to the limit matrix $X = \{x(i,j)\}$ maximizing the weighted entropy (Pittel 1967):

$$H = \sum_{i=0}^{n} \sum_{j=0}^{m} x(i,j) \ln \left[x(i,j)^{(0)}/x(i,j) \right]$$

under conditions

$$\sum_{i=0}^{n} x(i,j) = \bar{b}(j), \quad \sum_{j=0}^{m} x(i,j) = \bar{a}(i).$$

The matrix X (obviously) depends on the initial condition, i.e. on the choice of matrix X_0. Two different choices of X_0 seem to be reasonable:
1. As initial matrix X_0 the matrix $\mathfrak{A}$ is used, $x^{(0)}(i,j) = a(i,j)$.
2. As initial matrix the disequilibrium matrix $Q = \{q(i,j)\}$ of matrix $\mathfrak{A}$ with elements $q(i,j) = a(i,j)/[a(i)\cdot b(j)]$ is used.
Elements q(i,j) are equal to unity when alleles A(i) and B(j) are stochastically independent; otherwise q(i,j) are measures of dependence between A(i) and B(j). It has been proposed (Nijenhuis 1984) that in related populations disequilibria $\{q(i,j)\}$ will tend to a high degree of similarity.
We now show that both above mentioned choices of the matrix X_0 are equivalent, i.e. they both lead to the same limit matrix $X = \{x(i,j)\}$. Indeed, the solution maximizing

$$\tilde{H} = \sum_{i=0}^{n} \sum_{j=0}^{m} x(i,j) \ln \left[\tilde{x}(i,j)^{(0)}/x(i,j) \right]$$

where $\tilde{x}(i,j)^{(0)} = x(i,j)^{(0)} \cdot \alpha(i) \cdot \beta(j)$, maximizes also

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Construction of Two Locus Haplotype HLA-A,B Matrices For Poorly Studied Populations

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A genetic investigation of relationship should be based upon well defined frequency matrices for corresponding haplotypes. Two locus HLA-A,B matrices, however, exist for only a few thoroughly studied populations (Baur 1984). On the other hand some "similarities" between different populations have been well established (Nijenhuis 1984). Thus, attempts are made to use these similarities in order to perform analyses for populations whose matrices are only partial known. This can be done in different ways, depending on the definition of "similarity". We will show that different procedures within a large class result in the same approximate matrix. A regular method for constructing approximate matrices is proposed.

Let $\mathfrak{A}$ be a well established two-locus haplotype matrix; the first locus A having alleles A(i) occurring with frequencies a(i)(i=0, 1,...,n), and the second locus B having alleles B(j) occurring with frequencies b(j)(j=0,1,...,m). The frequencies of haplotype A(i)B(j) will be designated as a(i,j). Then

$$\sum_{i=0}^{n} a(i,j) = b(j) \quad (j=0,1,\dots,m); \qquad \sum_{j=0}^{m} a(i,j) = a(i) \quad (i=0,1,\dots,n); \qquad \sum_{i=0}^{n} a(i) = \sum_{j=0}^{m} b(j).$$

A corresponding matrix X for another insufficiently studied population is only partly known: all the allele frequencies $\bar{a}(i)$, $\bar{b}(j)$ (i=0,1,...,n; j=0,1,...,m) but only part of the haplotype frequencies x(i*,j*) are estimated. The problem is to compose the approximate matrix X with some fixed frequencies x(i*, j*) which obeys the limitations

$$\sum_{i=0}^{n} x(i,j) = \bar{b}(j) \quad j=0,1,\dots,m; \qquad \sum_{j=0}^{m} x(i,j) = \bar{a}(i) \quad i=0,1,\dots,n; \qquad \sum_{j=0}^{m} \bar{b}(j) = \sum_{i=0}^{n} \bar{a}(i).$$

For this purpose we apply the so-called gravitational algorithm (known also as Deming-Stephan's or Shelekhovski's algorithm) (Pittel 1967) which has been used in problems of distribution of resources with limitations. At the first step the initial matrix $X_0 = \{x(i,j)^{(0)}\}$ of size n×m is chosen. The effect of variations

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If one makes the neutral assumption that in any one case there is an equal chance of the unknown father of the child being German or alien, then - depending on the ethnic distance between child and mother - one may assume with W ≧ 90% that the father is of alien origin in between 0.2 and 56.7% of the cases and that he is of German origin in between 1.5 and 62.2% of the cases. A prerequisite for the correctness of these figures is, of course, that the father of the child in question is in fact either German or Italian, Turk, etc.

Often it is difficult to tell from a child's appearance whether its father is e.g. Italian, Turk, Spanish, etc. In these cases one should try to establish the probability of alienness of the father from the blood group findings for mother and child. This may help the court to reach a decision in keeping with the truth of the situation.

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pothesis X the child's father is alien, according to hypothesis Y German. The frequency of the former will then be

$f(X) = a_1 \cdot a_1$ (= frequency of the maternal genotype) $\cdot\, a'_1$ (= frequency of the gene the child has inherited from the father)

$= 0.9 \cdot 0.9 \cdot 0.1 = 0.081$,

and the frequency of the latter

$f(Y) = a_1 \cdot a_1 \cdot a_1$

$= 0.9 \cdot 0.9 \cdot 0.9 = 0.729$.

The probability of hypothesis X is thus

$W_X = f(X)/f(X)+f(Y) = 0.081/(0.081+0.729) = 0.1$.

Hence, in this case there is a probability of 10% that a child with the genotype aa will have an alien father; the probability of a German father is 90% - conditional on a neutral prior probability.

To combine the results from several systems one multiplies the likelihood ratios $L_1, L_2, \ldots, L_n$:

$f(Y_1)/f(X_1) \cdot f(Y_2)/f(X_2) \cdot \ldots \cdot f(Y_n)/(X_n)$.

The probability of hypothesis X (in %) tells one how often one would be correct if in 100 analogous cases one categorically decided in every case that the child's father was alien; the probability of hypothesis Y tells one how often one would be correct if in every case one assumed the father of the child was German (given the assumption of a neutral prior probability). -

The greater the frequency differences between the two populations concerned the clearer the indication of the alien father's ethnicity. Decisions are possible from W = 90% upwards.

One will calculate W_X or W_Y only if there is a prospect of obtaining a reasonably high W value (e.g. $W \geq 90\%$). In other words, before applying this method one should consider the conditions of the case in question, taking account of a) the ethnic situation and b) the type and number of gene systems included.

Because the lgY/X values for German and alien fathers do not have a normal distribution, and because the number of cases dealt with in practice is as yet too small, one must of necessity resort to simulation using adequate gene frequencies. We have simulated between 2000 and 4000 mother-child doublets in 15-21 systems. Table 1 gives the results.

Table 1. Proportion (in %) of triplets with German and alien father respectively, for which, with $W \geq 90\%$, the German or alien origin of the father was established from the lgK difference between mother and child (2000-4000 simulated triplets in 15-21 systems).

ethnic origin of the father	cases in favour of an alien father	cases in favour of a German father	mother-child doublets	number of systems
Italian	0,2%	1,5%	3000	21
Turkish	0,4%	4,4%	3000	21
Spanish	2,8%	2,5%	4000	20
Indian	2,8%	7,6%	3000	15
Arab	9,1%	13,4%	3000	18
urban Mexican	18,5%	15,7%	3000	18
USA Black	56,7%	62,7%	2000	19

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In all cases the lgK values of the children are clustered in the range between those of their respective parents (in accordance with known distribution curves).
The smaller the phenotype distance between the parents lgK values the greater the likelihood that the lgK values of their children will scatter beyond this range, and vice versa. As this "phenotype distance" increases so does the lgK distance of children from the lgK value of the mother. The lgK values of the children become increasingly indicative of an alien father; accordingly, the probability of a German father decreases.

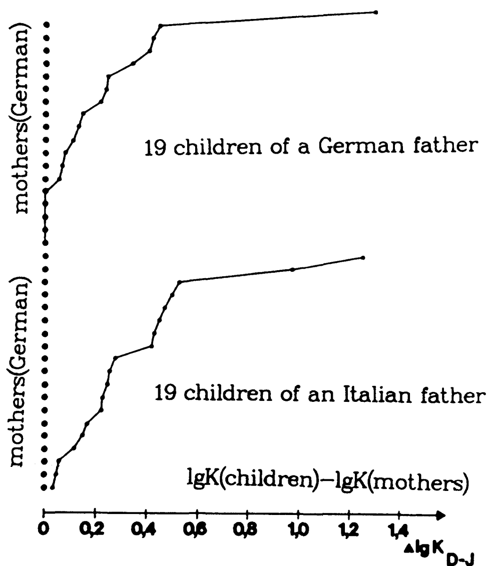

Fig. 2. Differences between the lgK values of child and German mother for 19 children with a German and 19 children with an Italian father.

Fig. 2 shows that the lgK differences between mother and child, on the whole, are greater if the father is Italian.

This behaviour is useful in cases of putatively alien paternity, for it opens up the possibility of determining the numerical probability of such parentage from findings for the child.

The mathematical basis of this phenomenon can be demonstrated with the example of a single blood group system.
The blood group system may include the combinant genes *1 and *2 as well as 3 phenotypes (= genotypes): 1, 2-1 and 2. Let us assume the following gene frequencies for the German and alien populations:

genes	frequencies German	alien
*1	a1 = 0.9	a'1 = 0.1
*2	a2 = 0.1	a'2 = 0.9

Let mother and child possess the genotype 1-1. According to hy-

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Seroanalysis as a means of establishing a child's unknown, putatively alien paternity

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A child either takes after its father or its mother or lies somewhere in between. The same applies to a child's blood group properties, i.e. a child's K value [1] may lie closer to that of the father or that of the mother or somewhere in between (Hummel 1986). If we proceed from defined triplets with a German and an alien father respectively, the lgK values of the children will be scattered symmetrically around those of their parents, provided that the latter are ethnically similar. As the "phenotype distance" between the parents increases there is a shift in the lgK values of the children away from those of the mother towards those of the father. Thus, if a child has an alien father this will be revealed by a blood group analysis: the child's lgK value will be higher than that of its mother (fig. 1).

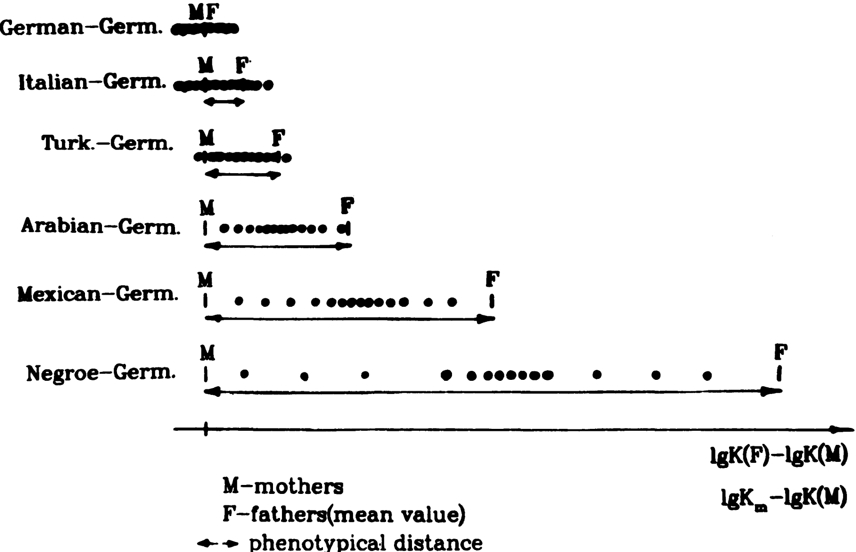

Fig. 1. Scattering of the lgK value of 14 children around the mean lgK values of their parents; in all cases a German mother, the fathers variously German, Italian, Turkish, Arab, urban Mexican and Black US American (schematic presentation).

[1] $K = f(II)/f(I)$, where f(II) is the frequency of a person's phenotype in the alien and f(I) that in the German population (cf. previous article).

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position of $\overline{\lg K_m}$. The mathematical analysis - which is beyond the scope of this paper - produced the following:

1. If gene systems are codominant, then $\overline{\lg K_m}$ will lie halfway between $\overline{\lg K(I)}$ and $\overline{\lg K(II)}$. -
2. If a system includes recessive-dominant genes, then $\overline{\lg K_m}$ will shift towards that population in which the dominant gene is more common. -
3. If a system includes a silent gene, then $\overline{\lg K_m}$ will shift towards that population in which this gene is rarer. -
4. If a system includes "complex" genes (e.g. Gm*1,2; Gm*1,b), then $\overline{\lg K_m}$ will shift towards that population in which non-complex genes are rarer. -
5. In a system with several non-codominant genes shifts in the same direction are cumulative, shifts in different directions neutralize one another.

The implications for seroanalysis are twofold:

I. Before a person can be assigned to one or other ethnic group one must examine all the available serological information. -

II. Statements about the portions of mixed blood in any specific person will be most realistic if only findings in codominant systems are included. Findings in systems with recessive-dominant and silent genes should be avoided unless these genes are more or less equally rare or common in both populations.

Table 1. Mean lgK values (theoretical) for Germans (G), USABlacks (USB) and their (mixed-blood) children (ch), from the findings in 17, 17+1 and 17+2 gene systems

systems	lgK(G)	lgK(USB)	lgK(ch)	mean dist. of ch. from the Europ. parent	mean dist. of ch. from the Black parent
n=17	-1.666	1.610	0.073	1.739	1.537
" +Fy(a,b,0)	-2.412	3.563	-0.173	2.239	3.736
" +Fy(a,b,0) +Gm(1,2,b)	-7.303	3.997	-0.148	7.155	4.145

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The following list shows the phenotype distances between Germans and other European and non-European populations:

popul.:	Yugo.	Ital.	Span.	Pers.	Turk.	Arab	urban Mex.	USA Black	W-Af. Black
no. of systems	17	19	20	17	22	17	18	19	23
phenot. dist.	0.223	0.380	0.407	0.557	0.658	2.003	2.090	11.300	17.844

The greater the phenotype distance, the less overlap there is between the lgK curves for the two populations, and hence the greater the certainty that an individual belongs to one or other population or, in the case of ethnically mixed blood, the more realistic are the seroanalytically determined blood portions. The smaller the phenotype distance between two populations the closer will W_{RI} and W_{RII} be to the given prior probability of 0.5.

The usefulness of W_{RI} and W_{RII} depends on how realistic their information is on the quantities of the mixed blood portions. To test the degree of reality of W_{RI} and W_{RII} we examined the influence of different gene systems on these values and the effects of the different phenotypes. For our model we used children of ethnically different parents.
As there were not enough cases from practice available for comprehensive studies we calculated theoretical and simulated lgK_m values for the filial generation.
One calculates children's phenotype frequencies m_i as follows: If an ethnically mixed person is homozygous AA, the one gene is inherited from the mother from population I [frequency: $p_1(A)$], and the other from the father from population II [frequency: $p_2(A)$]. The frequency of the genotype AA will then be $p_1(A)\ p_2(A)$. Correspondingly, the frequency of the heterozygous genotype AB will be

$$p_1(A)\cdot p_2(B)+p_2(A)\cdot p_1(B).$$

A phenotype can have one or more genotypes. The sum of their frequencies is the frequency of their phenotype. Accordingly, the mean lgK_m value in a specific gene system with n phenotypes will be

$$\overline{lgK_m} = \sum_{i=1}^{n} m_i \cdot lg\left[(f_i(II)/f_i(I)\right].$$

As the figures in Table 1 (see below) show, the mean lgK value for Black-White children (with findings in 17 systems) lies almost exactly between the mean lgK values for their parents. If one includes the findings in the Duffy system - because of Fy^O this system differentiates effectively between black and white, as shown in the greater phenotype distance between the parents - the mean lgK value for the mixed blood children shifts in the direction of the white parent. If one includes, in addition, the findings in the Gm system - because of the negroid complex gene Gm*1b - this further increases the phenotype distance between Black and White (= "parents") - the mean lgK value for the children shifts markedly towards the black parent.

These results encouraged an attempt to establish some rules in accordance with which the different gene systems influence the

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The "phenotype distance" can be more clearly demonstrated by complete lgK curves. One proceeds from a normal distribution and calculates the variance with the following formulae:

$$\text{var lgK(I)} = \sum_{i=1}^{n} f_i(\text{I}) \left\{ \lg \left[f_i(\text{II})/f_i(\text{I}) \right] - \overline{\text{lgK(I)}} \right\}^2;$$

$$\text{var lgK(II)} = \sum_{i=1}^{n} f_i(\text{II}) \left\{ \lg \left[f_i(\text{II})/f_i(\text{I}) \right] - \overline{\text{lgK(II)}} \right\}^2.$$

Figs 1 and 2 compare the distributions of the lgK values of 119 Germans and 51 Italians respectively as well as of 2000 simulated Germans and 2000 simulated Italians with the respective theoretical lgK distribution curves. The agreement between the theoretical curves and, in particular, the simulated values is so close that it appears permissible to use the relatively easily constructed theoretical curves in comparable studies (their mean values and variance are, in any case, reliable).

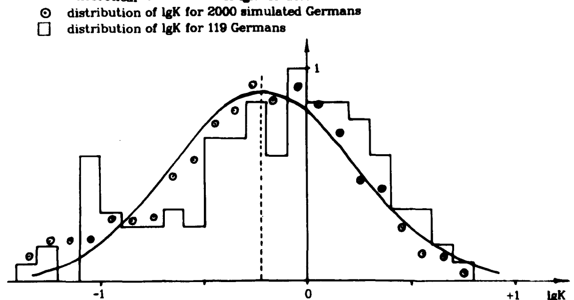

Fig. 1. Theoretical lgK distribution and mean value for Germans (in comparison with Italians), distribution of the lgK values for 2000 simulated Germans and for 119 random Germans (findings in 19 systems).

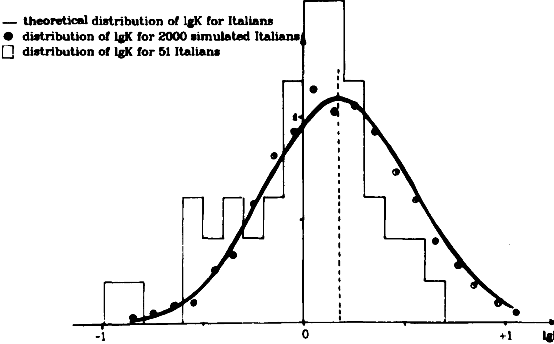

Fig. 2. Theoretical lgK distribution and mean value for Italians (in comparison with Germans), distribution of the lgK values for 2000 simulated Italians and for 51 random persons of Italian origin (findings in 19 systems).

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Empirical and theoretical studies on "seroanalysis"

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"Seroanalysis" is a mathematical method by which the population or - in the case of mixed blood - populations to which a person belongs can be established from his or her serotype (Hummel 1980, 1985, 1986). The person in question belongs either to population I or population II, or owns properties from both. If f(I) is the frequency of the phenotype of the person in population I and f(II) that in population II, then K is the likelihood ratio:

$$K = f(II)/f(I).$$

If the person belongs to population I, K will be very small, if to population II, K will be large. -
In practice, the great advantage of seroanalysis lies in the possibility it offers of determining the racial portions, R_I and R_{II}, in a person of mixed blood. This is done by calculating the probabilities

$$W_{RI} = 1/(1+K) \quad \text{(= blood portion from population I), and}$$

$$W_{RII} = 1/(1+1/K) = 1-W_{RI} \quad \text{(= blood portion from population II).}$$

The formulae are related to those of Essen-Möller (1938) and correspond to Bayes' Theorem (1763) with a neutral prior probability.

The greater the distance between the two populations, the more realistic are the statements W_{RI} and W_{RII} for a I-II-mixed-blood person.

To obtain mean lgK values one can use either the findings for typed individuals or calculate theoretical values from phenotype frequencies:

$$\overline{\lg K(I)} = \sum_{i=1}^{n} f_i(I) \cdot \lg\left[f_i(II)/f_i(I)\right] ;$$

$$\overline{\lg K(II)} = \sum_{i=1}^{n} f_i(II) \cdot \lg\left[f_i(II)/f_i(I)\right] .$$

$f_i(I)$ and $f_i(II)$ $(i=1,2,\dots,n)$ are the phenotype frequencies of a system in populations I and II respectively. If - as is usual - several systems are included one sums the mean values. -
Given the mean lgK values for both populations one is able to define the "phenotype distance":

$$D = \overline{\lg K(II)} - \overline{\lg K(I)} = \sum_{i=1}^{n} \left[f_i(II)-f_i(I)\right] \cdot \lg\left[f_i(II)/f_i(I)\right] .$$

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```
Übersicht für FallNr: 1.0       Fallname: XXXXXXXXXXXX
-------------------------------------------------------------------
    System              PI          EM       W(.5)
    1.0   A1A2B0      1.5731      9.8033   61.14%
    3.0   MNSs        1.6600      9.7799   62.41%
    4.0   KELL        1.0409      9.9826   51.00%
    6.0   Fy(a,b,0)   2.1796      9.6616   68.55%
    ...    ...         ...         ...      ...
   45.1   PLG(1,2,F   0.7543     10.1225   43.00%
-------------------------------------------------------------------
Endergebnis für FallNr: 1.0       Fallname: XXXXXXXXXXXX
    23 Systeme mit Befunden.
 Keine Systeme mit einem Ausschluss.
    23 Systeme wurden berücksichtigt.
L= 0.00161755    PI= 618.2207     EM=  7.2089    W(.5)= 99.8385%
  lg X= -21.8358     lg Y= -24.6270
    Proband                 Anzahl Systeme   lg Phänotypfrequenz
  1 Herkules                      23                -8.3140
  2 Alkmene                       23                -8.3734
  3 Zeus                          23                -9.7760
```

Abbildung 2.   Ausschnitte aus der Summenausgabe (KMV-Fall)

VORTEILE

Die Hauptvorteile einer computergestützten Auswertung sind unter anderem die Verläßlichkeit und Reproduzierbarkeit. Aber neben diesen generellen Vorteilen erweitert mPAPS auch das Methodenspektrum.
Bei einfachen KMV-Fällen erspart eine Computerauswertung das Nachschlagen in Tabellenbänden, Handrechnungen und das mühsame Einbringen der Statistik in das Gutachten.
Bei Verwandtenfällen kann der Gutachter verschiedene Hypothesen über mögliche Verwandtschaftsbeziehungen testen und sich so ein genaues Bild von dem Fall verschaffen.
Bei beiden Fallarten, KMV-Fall und Verwandtenfall, kann eine eventuelle Fremdstämmigkeit leicht berücksichtigt werden.
Die Lücke zwischen den Möglichkeiten im Labor (Subtypisierung, neue Erbsysteme) und der Biostatistik wird kleiner, da alle Daten selbst eingegeben werden können.
Eine Computerauswertung im eigenem Labor ermöglicht ein schnelles Erstellen eines Gutachtens.

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Probanden, die Wahrscheinlichkeit der Teilstammbäume, die Wahrscheinlichkeit für X und Y, der Likelihoodquotient, der Paternity-Index, der Essen-Möller-Wert und die Verwandtschaftswahrscheinlichkeit.
Bei beiden Fallarten können den Beteiligten verschiedene Populationen zugeordnet werden (Fälle mit Beteiligung Fremdstämmiger).
Zusätzlich zur Standardausgabe (Abb. 1) kann mPAPS eine summarische Aufstellung der Resultate erzeugen (Abb. 2). Ebenso kann ein Protokoll der verwendeten Systeme und Frequenzen angefordert werden.

```
      ...           ...           ...
------------------------------------------------------------------
SystemNr: 1.0       Systemname: A1A2B0
L= 0.6357       PI= 1.5731        EM=  9.8033      W(.5)= 61.14%
  lg X=  -0.7870     lg Y=  -0.9837
 Proband                     Phänotyp            Frequenz
  1 Herkules               (1)   0                 40.41%
  2 Alkmene                (1)   0                 40.41%
  3 Zeus                   (1)   0                 40.41%
  A=  4.50%    NA= 95.50%   lg NA=  -0.0200  lg p(KM)=  -0.5902
mögliche Gene= 0
------------------------------------------------------------------
SystemNr: 3.0       Systemname: MNSs
L= 0.6024       PI= 1.6600        EM=  9.7799      W(.5)= 62.41%
  lg X=  -1.8092     lg Y=  -2.0293
 Proband                     Phänotyp            Frequenz
  1 Herkules               (4)   MNss              22.42%
  2 Alkmene                (7)   Nss               13.85%
  3 Zeus                   (4)   MNss              22.42%
  A= 30.09%    NA= 69.91%   lg NA=  -0.1554  lg p(KM)=  -1.3798
mögliche Gene= *Ms

      ...           ...           ...
==================================================================
Endergebnis für FallNr: 1.0       Fallname: XXXXXXXXXXXX
    23 Systeme mit Befunden.
 Keine Systeme mit einem Ausschluss.
    23 Systeme wurden berücksichtigt.
L= 0.00161755    PI= 618.2207      EM=  7.2089    W(.5)= 99.8385%
  lg X= -21.8358     lg Y= -24.6270
    Proband                Anzahl Systeme   lg Phänotypfrequenz
  1 Herkules                     23                -8.3140
  2 Alkmene                      23                -8.3734
  3 Zeus                         23                -9.7760
  A= 99.4910%  NA=  0.5090% lg NA=  -2.2933  lg p(KM)= -14.8510
  a priori:    .1= 98.565%  .2= 99.357%  .3= 99.624%  .4= 99.758%
  .5= 99.839%  .6= 99.892%  .7= 99.931%  .8= 99.960%  .9= 99.982%
------------------------------------------------------------------
Kind-Mutter-Statistik:
L= 0.0145733     PI= 68.61871      EM=  8.1636    W(.5)= 98.5636
```

Abbildung 1. Ausschnitte aus der Standardausgabe (KMV-Fall)

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Diese Textdateien können von (fast) allen Textsystemen erzeugt und verändert werden. Die Verwendung von Texteditoren als Eingabeprogramm hat einige Vorteile. Die Eingabe eines Falles kann dann z.B. durch Einkopieren eines Musterfalles erfolgen. Nur sich ändernde Daten -Fallnummer, Namen der Probanden, Befunde usw.- müssen dann noch überschrieben werden. So ist der Aufwand, gemessen in Anschlägen auf der Tastatur, minimal.
Eine Datei, z.B. mit den Daten eines neues Blutgruppensystems kann leicht ausgetauscht werden, indem diese Datei auf eine Diskette kopiert und verschickt wird.

Ausgaben

Die von dem Statistikpaket mPAPS erzeugten Ausgaben werden nicht sofort auf einen Drucker geschickt, sondern in Dateien abgelegt. Damit stehen sie für eine unbeschränkte Weiterverarbeitung zur Verfügung. Sie können mehrfach ausgedruckt werden oder in ein Gutachten eingeflochten werden. Sie können zusammen mit dem Gutachten archiviert werden, z.B. indem sie auf eine Diskette gespeichert werden. Auch die Archivierung aller für die Auswertung relevanten Daten zusammen mit der Auswertung selbst ist damit leicht möglich.

Realisation

Bei der Realisierung eines Programmes, welches nicht nur von erfahrenen Spezialisten benutzt wird, ist besonderer Wert auf die Stabilität zu legen. Alle Eingabedateien müssen einer rigorosen Prüfung auf Plausibilität unterzogen werden. Schon bei der Konzeption der Eingabestruktur ist darauf zu achten, daß die Eingaben später leicht geprüft werden können. Hinweise und Fehlermeldungen des Programmes sollten möglichst eindeutig auf Probleme hinweisen und leicht verständlich sein.

STATISTISCHE METHODEN

Ein Statistikpaket für die serostatistische Abstammungsbegutachtung wird gemessen an der Fähigkeit Abstammungsfälle sachgerecht auszuwerten. mPAPS kennt (im Moment) zwei Fallarten: den Kind-Mutter-Vater-Fall und den Verwandtenfall. Für den Kind-Mutter-Vater-Fall werden in jedem Erbsystem folgende Werte berechnet: die Phänotypfrequenz der Probanden, die Wahrscheinlichkeit für X und Y, die Aus- und Nichtausschlußwahrscheinlichkeit, der Likelihoodquotient, der Paternity-Index, der Essen-Möller-Wert und die Vaterschaftswahrscheinlichkeit.
Bei einem Verwandtenfall werden zwei Hypothesen über (beliebige) Verwandtschaftsbeziehungen gegenübergestellt. Der Algorithmus von mPAPS ist sehr schnell, so daß auch komplexe Verwandtenfälle ausgewertet werden können. Im Augenblick darf ein Verwandtenfall 20 Probanden umfassen. Für einen Verwandtenfall werden in jedem Erbsystem folgende Werte berechnet: die Phänotypfrequenz der

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Ein Statistikpaket für die Abstammungsbegutachtung

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EINLEITUNG

Die weite Verbreitung und das günstige Preis/Leistungverhältnis von Personal-Computern ermöglichen die biostatistische Abstammungsbegutachtung mit dem Mikrocomputer. Die im Vergleich zur Groß-EDV einfache Bedienung und ständige Verfügbarkeit lassen es sinnvoll erscheinen, die bisher Großrechnern vorbehaltenen serostatistischen Auswertungen hin zum Gutachter zu verlagern. Diese Aspekte waren der Anlaß, das Statistikpaket mPAPS (micro-computer Parental Analysis ProgramS) für Personal-Computer zu entwickeln.

KONZEPT

Ziel war es, ein Programmsystem zu entwickeln, welches den Anforderungen als tägliches Arbeitsmittel gerecht wird. Dieses System sollte ferner offen sein, um spezielle Wünsche leicht berücksichtigen zu können. Vorhandene Mittel, z.B. Textsysteme, sollten benutzbar sein.

Daten

Alle notwendigen Daten werden nicht in einer Datenbank mit unzugänglichen Codierungs- und Speicherverfahren abgelegt, sondern in ASCII-Textdateien. Dateien können mit Programmen des Betriebssystems kopiert, gelöscht, archiviert, ausgedruckt oder in anderer Weise behandelt werden.
Ein Blutgruppensystem mit System-, Allel- und Phänotypnamen, mit der Zuordnung der Allele zu Phänotypen und mit Allelfrequenzen von verschiedenen Populationen wird in einer Datei zusammengefaßt. Eventuell verwandte Systeme, entstanden z.B. durch andere Subtypen, können in der gleichen Datei abgespeichert werden.
Ebenso wird ein Fall mit allen Angaben, zu den Personen und ihren Befunden in einer Datei abgespeichert. Sind z.B. für einen Verwandtenfall mehrere Annahmen über Verwandtschaftsbeziehungen zu prüfen, so können diese Variationen in der gleichen Datei abgelegt werden.

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$$L_X = \sqrt[N]{E_{KMV}(L|H_X)}, \qquad L_Y = \sqrt[N]{E_{KMV}(L|H_Y)} \quad .$$

Für verschiedene Werte von N erhalten wir Tabelle 4.

Tabelle 4. Beitrag eines Alleles zur biostatistischen Leistung

N	2	3	4	5	6	7	8
L_X	.9014	.8571	.8392	.8345	.8355	.8391	.8436
L_Y	.9405	.8986	.8685	.8559	.8518	.8519	.8541

Die optimale Anzahl Allele in einem System mit Gleichverteilung der Allelwahrscheinlichkeiten ist 5 für $E_{KMV}(L|H_X)$ und 6 für $E_{KMV}(L|H_Y)$. Angenommen wir hätten die Wahl zwischen einem System mit N=15 Allelen und drei Systemen mit je N=5 Allelen, so erhalten wir die Resultate der Tabelle 5.

Tabelle 5. Vergleich der Kennziffern

	N=5	$(N=5)^3$	N=15
$E_{KMV}(L|H_X)=\bar{A}$	0.4048	0.0663	0.1364
$E_{KMV}(L|H_Y)$	0.4593	0.0969	0.1454

Der Erwartungswert $E_{KMV}(L|H_X)=\bar{A}$ den Putativ-Vater nicht auszuschließen, ist mit den drei Systemen ($\bar{A}$=6.6%) nur halb so groß wie in dem einem System mit 15 Allelen ($\bar{A}$=13.6%).

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Tabelle 2. Die verschiedenen Likelihoodquotienten

Typ	Likelihood	Kombination von Tabelle 1
I	$L = P_i$	T1 und T2.3
II	$L = 2P_i$	T1 und T2.3
III	$L = P_i+P_j$	T2.1
IV	$L = 2(P_i+P_j)$	T2.2

In einer längeren Handrechnung fassen wir die Gewichte für X bzw. Y dieser vier Likelihoodquotienten zusammen und erhalten Tabelle 3.

Tabelle 3. Formeln zur Berechnung der Leistungskennziffern

Typ	Summe	Gewichte für Y	Gewichte für X	L
I	$\sum_{i=1}^{N}$	$P_i^3(1-P_i+P_i^2)$	$P_i^2(1-P_i+P_i^2)$	P_i
II	$\sum_{i=1}^{N}$	$2P_i^2(1-P_i)(1-P_i+P_i^2)$	$P_i(1-P_i)(1-P_i+P_i^2)$	$2P_i$
III	$\sum_{i=1}^{N-1}\sum_{j=i+1}^{N}$	$P_iP_j(P_i+P_j)^3$	$P_iP_j(P_i+P_j)^2$	P_i+P_j
IV	$\sum_{i=1}^{N-1}\sum_{j=i+1}^{N}$	$2P_iP_j(P_i+P_j)^2(1-P_i-P_j)$	$P_iP_j(P_i+P_j)(1-P_i-P_j)$	$2(P_i+P_j)$
	N(N+1)	$\Sigma^*Y = \bar{A}$	$\Sigma^*X = 1$	

Die Formeln aus der Tabelle 3 ermöglichen eine schnelle Berechnung beliebiger Leistungskennziffern. Dabei müssen nur N(N+1) Produkte berechnet und summiert werden.

OPTIMALE GESTALT EINES SYSTEMS

Optimale Verteilung der Allelwahrscheinlichkeiten

Wir beschränken uns im folgenden auf Leistungskennziffern für den KMV-Fall. Wie schon bekannt, z.B. Selvin (1980), ist die Ausschlußwahrscheinlichkeit in einem kodominanten Erbsystem am größten, wenn die Allele gleichwahrscheinlich sind. Entsprechend haben $E_{KMV}(L|H_X)=\bar{A}$ und $E_{KMV}(L|H_Y)$ bei gleichverteilten Allelen ein Minimum.

Optimale Anzahl der Allele

Wir stellen die Frage, ob es im biostatistischen Sinne günstiger ist, ein System mit 'vielen' Allelen oder mehrere Systeme mit 'wenigen' Allelen zu verwenden. Dazu setzen wir eine Gleichverteilung der Allelwahrscheinlichkeiten voraus, $P_i=1/N$, und berechnen den Beitrag eines Alleles zur Leistungskennziffer mit

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Tabelle 1. X, Y und L für den KMV-Fall

	M V	Y=p(KM)p(V)		X	L
	K	p(KM)	p(V)	p(KMV)	Y/X
T1	i-m i-v i-i	$M_iM_mU_i$	$d_{i-v}V_iV_v$	$M_iM_mV_iV_v$	$d_{i-v}U_i$
T2.1	i-j i-j i-j i≠j	$M_iM_jU_i+$ $M_iM_jU_j$	$2V_iV_j$	$2M_iM_jV_iV_j$	U_i+U_j
T2.2	i-j j-v i-j i≠j, i≠v	$M_iM_jU_i+$ $M_iM_jU_j$	$d_{j-v}V_jV_v$	$M_iM_jV_jV_v$	$d_{j-v}(U_i+U_j)$
T2.3	i-m j-v i-j i≠j, j≠m	$M_iM_mU_j$	$d_{j-v}V_jV_v$	$M_iM_mV_jV_v$	$d_{j-v}U_j$

LEISTUNGSKENNZIFFERN FÜR DEN KIND-MUTTER-VATER-FALL

Einführung

Die Erwartungswerte biostatistischer Kennziffern eines Falltyps kennzeichnen die Leistung eines Blutgruppensystems. Wir betrachten nur den KMV-Fall und exemplarisch als Kennziffer den Likelihoodquotienten L. Für die Berechnung des Erwartungswertes des Likelihoodquotienten L müssen wir jeden Wert L(KMV) mit der Wahrscheinlichkeit seines Auftretens gewichten. Die Wahrscheinlichkeit einer KMV-Terzette ist unter den Hypothese H_X bzw. H_Y verschieden. Wir notieren die bedingten Erwartungswerte mit $E_{KMV}(L|H_X)$ resp. $E_{KMV}(L|H_Y)$. Diese Erwartungswerte werden durch Summation Σ^* über alle genetisch möglichen KMV-Terzette berechnet.

$$E_{KMV}(L|H_X) = \Sigma^* X(KMV)L(KMV) = \Sigma^* XY/X = \Sigma^* Y = \overline{A} = E_{KMV}(\overline{A})$$

$$E_{KMV}(L|H_Y) = (\Sigma^* Y(KMV)L(KMV))/\Sigma^* Y(KMV) = (\Sigma^* Y^2/X)/\overline{A}$$

Die Summe $\Sigma^* X(KMV)$ ergibt Eins. Die Summe $\Sigma^* Y(KMV) = \Sigma^* p(KM)p(V)$ ist die mittlere Nichtausschlußwahrscheinlichkeit $\overline{A}$. Da diese i.a. ungleich Eins ist, müssen wir die Gewichte Y(KMV) durch Division mit $\overline{A}$ normieren.
Die obigen Aussagen gelten für beliebige Erbsysteme.

Numerische Durchführung

Zur numerischen Berechnung der Erwartungswerte können wir alle KMV-Terzette aufstellen, auf genetische Kompatibilität prüfen und auswerten. Aber wie wir Tabelle 1 entnehmen, gibt es in kodominanten Systemen unter der Annahme der gleichen Population P_i für alle Probanden nur vier verschieden gebildete Likelihoodquotienten (Tabelle 2).

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Vaterschaftstatistik in Erbsystemen mit N kodominanten Allelen

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BEZEICHNUNGEN

Wir betrachten ein System mit N kodominanten Allelen. Die Allele numerieren wir von 1 bis N durch und sprechen sie durch ihre Nummer an. Als Variable für diese Allelnummmer verwenden wir i,j,v,m. Einen Genotyp, bestehend aus den Allelen i und j, notieren wir durch i-j. Für einen Genotyp i-j definieren wir eine Funktion d_{i-j}, welche 1 ist für i gleich j (homozygoter Genotyp) und 2 für i ungleich j (heterozygoter Genotyp).

AUSWERTUNG DES KIND-MUTTER-VATER-FALLES

Bei der Auswertung eines KMV-Falles unterscheiden wir die folgenden Kombinationen:
T1 das Kind ist homozygot;
T2 das Kind ist heterozygot und
  T2.1 Kind, Mutter und Vater tragen den gleichen heterozygoten Genotyp,
  T2.2 nur Kind und Mutter tragen den gleichen heterozygoten Genotyp, einen davon verschiedenen trägt der Vater,
  T2.3 Kind und Mutter tragen verschiedene Genotypen, der Genotyp des Vaters ist beliebig.
Für diese Möglichkeiten berechnen wir in Tabelle 1 die Wahrscheinlichkeiten für die Kind-Mutter-Dublette p(KM), für den Phänotyp des Vaters p(V), für Y=p(KM)p(V) und X=p(KMV). Die Probanden Mutter M, unbekannter Erzeuger U und der Vater V entstammen möglicherweise verschiedenen Populationen. Die Wahrscheinlichkeit eines Allels i bezeichnen wir deshalb mit M_i, U_i oder V_i. Die letzte Spalte der Tabelle 1 enthält den Likelihoodquotienten L=Y/X. Wie wir sehen, spielt die Population der Mutter M und des Vaters V keine Rolle. Der Likelihoodquotient L wird ausschließlich gebildet mit den Frequenzen des unbekannten Erzeugers U. Diese alleinige Abhängigkeit von der Population des unbekannten Erzeugers ist weithin nicht bekannt. Mit den Formeln der letzten Spalte von Tabelle 1 ist es leicht, Befunde einer KMV-Terzette auszuwerten.

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The significance of this observation is that since significant numbers of undisclosed uncles were found among excluded men with AI $\geqslant$ 4, not only are significant numbers of undisclosed uncles expected among excluded men with AI < 4, but also among non-excluded men. Thus, while one always considers that the tested man might be the father or a random man, one should also keep in mind the possibility that he might be an uncle; this additional possibility bears on the issue of adequacy of testing. The quotient PI/AI tests the hypothesis that the tested man is the father <u>versus</u> the hypothesis that he is an uncle. As shown in Table 2, the distribution of the cumulative PI among the genetic systems significantly affects AI and, therefore, PI/AI.

Table 2. The effect of the distribution of evidence among genetic systems on AI and PI/AI; cumulative PI = 64 in 6 systems

Individual PI in system							
#1	#2	#3	#4	#5	#6	AI	PI/AI
2	2	2	2	2	2	11.4	5.61
4	4	4	1	1	1	15.6	4.10
64	1	1	1	1	1	32.5	1.97
256	1/2	1/2	1	1	1	72.3	0.89

Note that if the PI is distributed evenly among the genetic systems, AI is small compared to PI. However, in the limiting case in which all the evidence in favor of paternity is found in a single system, with the remaining systems on balance favoring non-paternity, AI>PI; the genetic evidence actually favors the hypothesis that the man is an uncle over the hypothesis that he is the father. In such a case the assumption that non-paternity implies that the tested man is "random" is critical.

THE INCEST INDEX

The incest index (II) tests the hypothesis that the father is a first order relative (brother, father) of the mother relative to the hypothesis that the father is unrelated to the mother. If Z is defined as the frequency with which a child of the observed phenotype is produced by a woman of the phenotype of the mother and a man <u>of the genotype of the mother</u>, and Y is calculated by "Method B", then for an individual genetic system II = (Z+Y)/2Y. II depends only on phenotypes of mother and child and can easily be extended for any degree of kinship. For our test battery, significant values of the II are due primarily to situations in which mother and child are HLA identical, or the child is apparently homozygous in HLA for a maternal haplotype. The use of the II in our clinical practice will be presented elsewhere.

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To test this hypothesis, followup was requested on the 19 cases (5.3%) with AI ⩾ 4 (Table 1). The yield from this population at increased risk for undisclosed unclehood is thus 6/18 (33%); the frequency with which this limited followup resulted in disclosure of related men (6) is twice the frequency with which mothers initially alleged unclehood (3). To investigate the distribution of the AI among uncles our files were reviewed.

Of 6500 cases (including the 1500 which constitute the prospective study reported here) the mother initially named related men in fifteen. In thirteen, she named a pair of brothers; in one case both brothers were excluded and in another case neither brother was excluded. In one case the mother named three brothers, and in one case the mother named three brothers for each of two children. After testing, a total of 18 alleged uncles remained, of whom 17 were confirmed as uncles; the distribution of the AI for these 17 is shown in Figure 1. While it is hazardous to estimate the shape of the distribution from such a small sample, the median of the distribution (7.7) may be reliably estimated (range of AI: 0.2-680). Thus, the distribution of the AI for uncles appears to be shifted by a factor of 30 from the distribution of excluded men (which is comprised primarily of random men). The distribution of AI's for uncles corresponds well to the inflection in the distribution of excluded men. As a significant number of undisclosed uncles were discovered among the 19 excluded men with AI ⩾ 4, one can expect that significant numbers of undisclosed uncles remain among the excluded men with AI < 4.

The distributions of the AI for excluded men and for uncles are not strictly comparable. In our laboratory we test sequentially. All our cases are tested in HLA, ABO, Rh, and MN; the great majority are also tested in PGM, EsD, and Glo. If clearcut exclusion or a PI > 100 is obtained, no further testing is performed. Otherwise sequential testing is continued in systems selected from AcP, AK, ADA, 6-PGD, Hp, Bf, PLG, Gc, Ss, Kell, Duffy, Kidd, subtyping in PGM and Gc, and, where appropriate, CAII and Hgb. Most non-fathers are easily excluded and thus receive relatively little testing. On the other hand, uncles are rather difficult to exclude (for any genetic system, no matter how powerful for excluding random men, the exclusion probability for an uncle < 1/2) and received, on average, more testing. In fact, of the 18 uncles, only 14 (78%) were excluded by our battery of tests, which has a mean exclusion probability for random men of .997. Three were excluded by further testing in other laboratories and, as noted above, one was not excluded.

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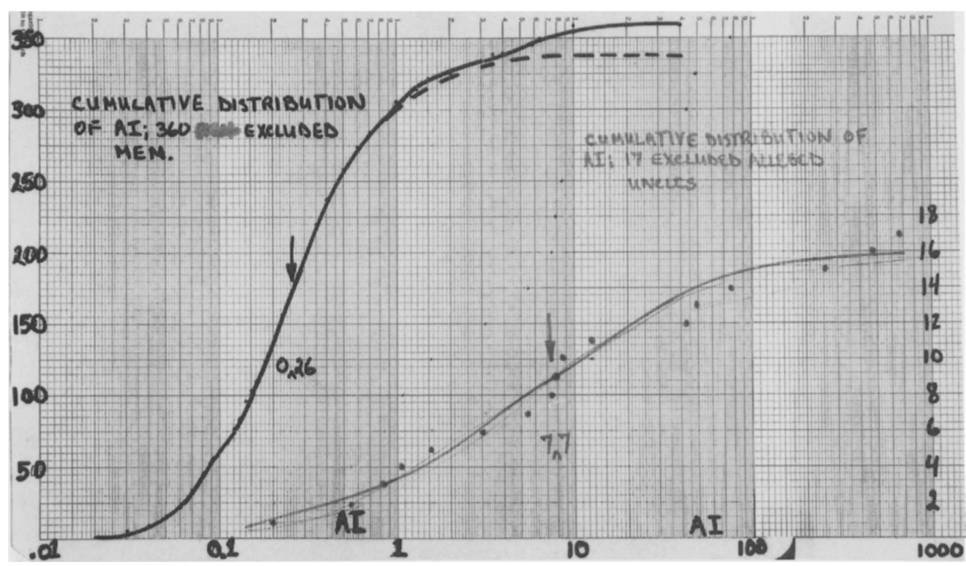

Fig. 1. Cumulative distribution of AI (logarithmic scale) for 360 excluded men and for 17 uncles excluded as the father.

Table 1. Followup of 19 excluded men with AI ≥ 4

- 1 no followup information obtained
- 1 alleged uncle[a] (7.5)
- 3 mother denies <u>any</u> other man (5.9, 5.9, 6.3)
- 8 mother admits to <u>unrelated</u> man
  - 1 tested (4.0); PI = 203
  - 7 not tested (4.7, 6.5, 7.5, 7.5, 23, 39, 40)
- 6 mother admits to <u>related</u> man
  - 2 brothers tested
    - 1 not excluded (23); PI= 36,900
    - 1 excluded[b] (6.1)
  - 4 not tested
    - 2 brothers (4.5, 10.2)
    - 1 cousin (6.1)
    - 1 uncle (5.7)

---

[a] of the three pairs of brothers initially named by mothers, one member of each pair was excluded. In one of these three cases, the AI of the excluded brother ("alleged uncle") was 7.5.

[b] Identity of this excluded brother has yet to be confirmed.

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It is clear that each gene in a zygote produced by the unphenotyped brother is "common" with probability 1/2 and "random" with probability 1/2. If a "common" gene is contributed, the probability that an unphenotyped mother would produce such a child with such a woman is X, while if a "random" gene is contributed, the probability that an unphenotyped mother would produce such a child with such a woman is Y. Since each of these situations occurs with equal probability, it follows that the probability that an unphenotyped brother of the tested man would produce such a child with such a woman is equal to (X+Y)/2.

The avuncular index (AI) is defined as the likelihood ratio which tests the hypothesis that the tested man is a paternal uncle of the child *versus* the hypothesis that the tested man is unrelated to the child, so that AI = (X+Y)/2Y = (PI+1)/2. The AI also tests the hypothesis that the tested man is a paternal half brother of the child (i.e. that the unphenotyped father of the tested man is the father of the child). By arguments similar to the above the likelihood ratio for an unphenotyped second degree relative (half brother, uncle, double first cousin) of the tested man can be shown to be equal to (X+3Y)/4Y = (PI+3)/4. This can easily be extrapolated to any degree of kinship.

While for a single genetic system the AI is a simple function of the PI, the cumulative AI is *not* a simple function of the cumulative PI. This point is of some practical importance and will be discussed later.

EXPERIMENTAL RESULTS

The AI was calculated in a prospective study of 1500 consecutive paternity cases. In 37 of these cases the mother named two men; 3 of these pairs of men were brothers, so that the prevalence of alleged unclehood in this material is one in 500. A total of 360 men were excluded, including at least one of each of the 37 pairs. The cumulative distribution of the AI for the 360 excluded men is shown in Fig. 1. The cumulative distribution is approximately sigmoidal, suggesting that this population of mostly random (with respect to their mother-child pairs) men have AI that are approximately log normally distributed. The median AI for the population is 0.26 - for the typical excluded man, his phenotypes occur four times more frequently among random men than among uncles of the respective children. Two inflection points are present, at AI $\sim$ 1 and AI $\sim$ 4. This suggests that the population of excluded men may not be homogenous; the distribution of random excluded men might well be represented by the dotted line, with the excess of excluded men with high AI's due in part to undisclosed uncles.

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The Avuncular Index and the Incest Index

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INTRODUCTION

From time to time in the paternity laboratory evidence for non-paternity is obtained in one or more genetic systems, while the remaining systems strongly suggest paternity. While this can occur by chance, such cases should be pursued further. Such a situation can be an important clue that silent alleles are present, if the evidence for non-paternity is limited to conditions of apparent opposite homozygousity (so-called "second order exclusions"). Rarely, such a situation can be a clue that the evidence for non-paternity is based on serologic irregularities; confirmation may be prudent. More commonly, especially when the evidence for non-paternity is incontrovertable, the situation provides a clue that a man related to the tested man is the father. We derive the theoretical framework for such an evaluation and review our experience with case material, which demonstrates that undisclosed unclehood is by no means rare.

DERIVATION OF THE AVUNCULAR INDEX

Suppose in a disputed paternity matter the results in a particular genetic system are such that the probability that a mating of a man of the phenotype of the tested man and a woman of the phenotype of the mother would result in a child of the observed phenotype is X, while the probability that a mating of an unknown (i.e. genetically random) man and a woman of the phenotype of the mother would result in a child of the observed phenotype is Y. This standard formulation yields a likelihood ratio (paternity index) of X/Y.

It is of interest to ask: what is the probability that an unphenotyped brother of such a man would produce such a child with such a woman? An unphenotyped brother of such a man has two sets of genes (haplotypes). One set is to be called "common" genes. Each "common" gene was inherited by both the tested man and his unphenotyped brother as part of the same chromosome segment from the same parent. The other set, to be called "random" genes, were inherited by the unphenotyped brother but not by the tested man.

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b. Practical inadequacies

* creation and inability to cope with "difficult cases";

* risk of misunderstanding of the expert's role in paternity investigation.

The only way to avoid these problems is to stick to the time honoured methodology introduced by ESSEN-MÖLLER and not to use, at least when dealing with non-experts, the exclusion concept.

In practice that means a generalization of the proposal of GÜRTLER (1977) to the cases where non-conformity of the genealogical hypothesis with the genetic model cannot be alleviated by the presence of a silent gene but only by mutation. That is to say: L or W can be calculated in any cases and they are the only statistics that can deal in a uniform, unbiased manner with all kinds of genetic results.

Another corollary of this proposition is that a coherent efficiency criterion of genetic analysis for paternity investigation can no longer rely on the exclusion probability, but on the difference between the means of paternity probabilities among fathers and non-fathers. The description of the derivation of the algorithm is deferred to another paper.

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Finally one could admit that even if the dichotomic approach was not, under the strict expert's point of view formally correct or practically advisable, it would be suitable for a satisfactory simplified version addressed to laymen. Unfortunately, in our opinion, this again is not true.

In fact, the formulation of the expert's opinion in terms of exclusion/non-exclusion has two serious drawbacks. On one hand, an opinion of exclusion of paternity, being given in categorical, non-probabilistic terms, gives the court the idea that the result is absolutely irrevocable. In more precise words, that no matter the progress in scientific knowledge or the future collection of further evidence on the case, the expert's opinion of exclusion would be the same, without gain or loss of confidence. Inversely, non-exclusion results being transmitted in a probabilistic form, it is legitimate to suppose that the court feels a rather different responsibility on the final decision in each type of cases.

Although this brief discussion would allow the immediate formulation of some conclusions, we think to be useful to devote a few lines to the recent controversies on the formulation of a statistic opinion.

Indeed, these controversies stem directly from the use of the dichotomic approach. We will take the proposition of LI and CHAKRAVARTY (1985) as the ultimate example of this strategy. These authors use a transformation of the *a posteriori* exclusion probability as a paternity probability (Pt), claiming to be superior to L or W. It has been already demonstrated that Pt is indeed poorer than L or W (BAUR *et al.*, 1986). We think that Pt is not only a less informative statistic when compared with L or W, but also that it is severely biased: the genetic information being only used in terms of exclusion/non-exclusion, (a) by definition, Pt is only usable in non-exclusion cases, preventing *a priori* its application to the opposite type of results. (b) all non-excluded men for the same mother/child pair have an equal Pt and (c) again by definition Pt cannot lower the established *a priori* value for paternity probability.

CONCLUSIONS

It is demonstrated that any methodology relying on the dichotomy exclusion/non-exclusion has serious and undesirable consequences in paternity expertises:

a. Formal inaccuracies

* non-uniformity of the statistic treatment of genetic data;
* incorrect assumption (or statement) that a paternity probability can assume a zero value (but not the unity).

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```
(2)         ┌Exclusion (Non-paternity; PP=0)
Phenotypic  │
 evidence  ─┤             statistical
            │              analysis
            └Non-exclusion ────────> Paternity Probability (0&lt;PP&lt;1)
```

We will call **non-dichotomic** to the first approach and, obviously, **dichotomic** to the last. In the next section we will analyse the assumptions of each of them in order to be able to formulate an opinion on their relative convenience for paternity expertises.

DISCUSSION

The first question to rise when comparing the dichotomic and non-dichotomic approaches is to ask the legitimacy of accepting that an experimental result can assume probability values of one or zero under no matter what hypothesis. We obviously assume the only scientifically admissible attitude: that any explanation is always provisional.

It can be argued however that the reliability of some exclusions can be *practically* taken as one. This opinion would only be (even *practically*) acceptable if the dichotomic approach had the following properties: (a)reasonably error free; (b)not biased; (c)simplification of the analysis and (d)improvement of the understanding of the expert's opinion by laymen.

The first property is well known not to be observed; indeed, false exclusions (namely of the 2nd. order type) are intolerably frequent. Some authors, acknowledging this fact, advanced some rules to minimize the risk of excluding a true father. For instance, according to VALENTIN (1977) at least two exclusions must be observed, while CHAKRABORTY and RYMAN (1981) suggest that in doubtful cases a paternity probability should be calculated for the systems where paternity is considered compatible. In any case, a non-uniform statistic treatment of the data is advised.

Thus it seems that the only way to avoid a high risk of error is to stick to a statistically biased solution, where different weights are given to evidence classes (exclusion and non-exclusion results).

Concerning the simplification eventually introduced by a dichotomic approach, one must admit that in favourable cases, the number and/or the type of observed exclusions allow a fast and safe production of the expert's opinion. However in a far from negligible proportion of the cases the concept of exclusion produces the opposite effect, as outlined above.

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THE BIAS OF THE DICHOTOMY EXCLUSION / NON-EXCLUSION AND THE EVIDENTIAL VALUE OF L (OR W)

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The aim of paternity expertises is to estimate the relative likelihood of mutually exclusive genealogical hypotheses, based on the results of a genetic analysis.

However, it is current practice to define it otherwise: the phenotypic evidence is the basis for a decision in terms of possible exclusion of one of the hypotheses; only if this aim is not attained, a statistical analysis is performed.

The purpose of this work is to demonstrate that this approach is not only grossly simplistic and inapplicable to a non negligible fraction of practical cases but also that it introduces implicitly a bias in the analysis of the problem, challenging the expert's neutrality.

DEFINITIONS

Let us call E to the **phenotypic evidence** obtained in paternity cases (blood group, enzymes, RFLPs, etc.). For these observations, a probability of occurrence can be calculated under the hypotheses of paternity (H1) or non-paternity (H2).

Thus a **paternity probability** (PP), has the form of a comparison between P(E/H1) and P(E/H2). For instance, in the **paternity index** (L), this comparison takes the simple form of a ratio.

The information flux in the process of paternity expertises is, therefore, the following:

(1)

$$\text{Phenotypic evidence} \xrightarrow{\text{statistical analysis}} 0 < \text{Paternity probability} < 1$$

Defining an **exclusion** as any E that contradicts the pre-established formal genetics hypothesis on the transmission rules, we will consider two types: a **1st. order exclusion** is said to occur when the hypothesis of paternity is not acceptable unless invoking mutation, whereas in the **2nd. order exclusion** the presence of a silent gene can also be presumed. When using the exclusion concept the information flux (1) is replaced by:

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Table 1

PI Calculated Without Single Indirect Exclusion Prior To HLA

Residual Index	Tested #	HLA Excludes #	HLA Excludes %
≤ 10	38	30	78.9
10-20	18	14	77.8
20-50	17	8	47.1
50-100	5	3	60.0
> 100	37	12	32.4
Total	115	67	58.3

Table 2

Results of Additional Testing In Cases With A Single Indirect Exclusion

System	# Cases	# HLA Excludes	Null Frequency
GC	5	5	0.000
F13A	2	2	0.001
BF	7	6	0.0009
HP	7	6	0.001
ACP	9	7	0.002
GLO	8	6	0.001
ESD	3	2	0.0007
GM	3	2	0.001
JK	13	8	0.001
RH	8	5	0.0007
F13B	2	1	0.001
MNSs	10	6	0.001
PI	1	1	0.0001
PLG	15	7	0.0035
PGM1	7	2	0.001
FY	15	1	0.005

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2) an exclusion is present (index = 0).
3) the child and tested man have a "null" allele.
4) the alleged father is a random man who may be either homozygous or has the "null" allele. (r/q+2r where r is frequency of null and q is frequency of the normal allele).

Table two shows by system the number of cases observed and excluded by additional testing and the gene frequency of silent alleles we use for our calculations. It is of interest that in all systems except three [FY (6.7%), PGMi (28.6%), and PLG (46.7%)], HLA establishes non paternity for 60% or more of the tested men. This probably reflects the greater frequency of nulls in these systems.

Depending on whether the PI calculated by method # 4 is above or below 20 various conclusions are provided. If the PI value $\geq$20 the report included the following:

1) Failure to exclude in all other genetic systems tested and calculations made considering various options with regard to the excluding system indicated almost with certainty that the tested man is the biologic father of the child in question.

2) Evidence of sexual intercourse between the mother of the child in question and the tested man at a time proximate to the conception of the child in question and ruling our sexual contact between the mother of the child in question and any first degree relative of the tested man could further strengthen the possibility that the tested man is the biologic father of the child in question.

   In cases in which the calculated value is less than 20 an additional statement is added to the above;

3) To prove with certainty whether the tested man has the rare null gene in the "excluding" system, family members would need to be tested. This may include parents, siblings, and any other children of the tested man.

This approach appears to be a reasonable solution to providing scientific data to the court in difficult cases. It also provides a basis for decisions on whether to pursue family studies.

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A Protocol for Reporting Single Indirect Exclusions.

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It is difficult to reach a conclusion with regard to paternity when the only inconsistency is reverse homozygosity in one system. The finding of a "single indirect exclusion" often occurs when a child and parent have a common null allele. On the other hand, in some cases this observation establishes non parentage. This report will present a protocol for reporting and resolving the question of parentage in such cases.

In testing 23,000 trios of disputed parentage we have observed exclusions of the alleged father in 30% of cases after routine testing (red cell antigens, serum proteins, red cell enzymes with CEP $\geq$.95). In a small percent of cases only a single indirect exclusion was found. In such cases a report is prepared which indicates the following:

1) the results do not provide sufficient information to establish whether or not the tested man may or may not be the biologic father.

2) an explanation is given of the results in the discrepant system.

3) a recommendation to test additional genetic systems including HLA is made.

4) Neither the Paternity Index (PI) or likelihood value is reported.

Table one summarizes 115 cases in which additional testing has been done. In 67 cases the additional testing (HLA) excluded the tested man. The original residual PI was less than 50 in 77% of excluded cases. This table indicates that the residual PI (PI calculated after assigning the excluding system a value of 1) is helpful in predicting whether additional testing will provide exclusionary evidence.

If the additional testing (CEP $\geq$.99) fails to exclude a second report is prepared which includes a set of calculations based on the following assumptions:

1) the index for the discrepant system equals 1 (residual PI).

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TABLE 1: Mean probability of exclusion ($\bar{A}$) for whites and blacks, with and without mother, for multiple genetic systems.

	Whites %(± SE 2)			Blacks %(± SE 3)		
System	n	With Mother	Without Mother	n	With Mother	Without Mother
*ABO	577	18	3	176	18	9
*MNSs	541	31	18	163	28	15
MN	571	19	12	172	22	14
*Rh	571	36	22	172	20	8
*Fy	538	26+	19+	161	16	2
*Jk	536	16	11	156	13	8
*Gm	468	26	12	116	22	13
*Hp	507	19	12	125	17	11
*HLA	585	93	85	174	93	84
*Combined Probability of Exclusion (CPE)		99	95		98	92

n=cases evaluated

+$\bar{A}$ values for Fy in whites slightly overestimate (1-2%) the exclusion probabilities due to the gene frequency of Fy which is approximately .0255 in this population. The resulting CPE values corrected for this variance are not affected.

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DISCUSSION

The results indicate that blood tests can be extremely useful in the exclusion of paternity using only the phenotypes of the AF and C. The most useful systems, depending on the race, were the HLA, Rh, Fy, MNSs, and Gm systems. The combined probability of exclusion (CPE) using these systems is approximately 90% for both races. The addition of more systems to the test panel can raise the CPE to over 92-95% depending upon the additional systems utilized. Clearly, the HLA system is very powerful in these tests and retains about 90% of the value obtained when the mother is included, in contrast to 12-70% for the other systems evaluated.

Formulas for estimating $\bar{A}$ of various blood group systems without testing the mother have been given by Lee, Lebeck and Pothiawala in 1980.[1] $\bar{A}$ values for 21 systems were calculated using gene frequencies of American whites. However, their report did not consider all of the potentially incompatible phenotypes of AF and C. In addition, only 5 HLA-A and 5 HLA-B markers were recognized in the HLA calculation.

Most of the $\bar{A}$ values derived in this study are in agreement with, or higher than those reported by Lee et al. More incompatible phenotypes were recognized in the Rh system and many more HLA A and B locus markers were identified in this study.

Whenever feasible, the mother's parents should be phenotyped since this additional information may, by itself, point toward an exclusion or alternatively, a higher PI. Blood tests of genetic markers of the alleged father and child can be very useful, even in the absence of the mother's specimen, in resolving cases of questioned paternity.

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Paternity Testing with an Absent Mother: The Probability of Exclusion of Red Cell Surface Antigen, Gm, Hp, and HLA Systems in North American Whites and Blacks

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INTRODUCTION

In some instances of questioned paternity, the laboratory may not have a blood sample from the mother. She may be unavailable because of death, mental illness, non-cooperativeness, intentional avoidance, or other problems. In such cases, the blood tests can still be used to either exclude the alleged father or derive a probability of paternity if there is a failure to exclude. This study was developed to examine the mean probability of exclusion ($\bar{A}$) of red cell surface antigen, Gm, Hp, and the HLA systems in North American whites and blacks in such cases.

METHODS

Paternity test cases completed between June, 1975 and April, 1987 were separated by the race of the adults into two groups - white and black. Mixed racial pairs and other racial groups were excluded from the study. The alleged father (AF) in each case was then moved forward one case in the sequence and re-assigned as the AF in the next mother(M)-child(C) combination in order to create false trios. The false trios were examined manually in duplicate to determine whether or not the AF could be excluded in any genetic system as the biological father of the child, either with or without including the phenotypes of the mother. Genetic markers identified included: ABO:A_1,A_2,B,O; MNSs:M,N,S,s: Rh:C,c,C^w,D,E,e; Fy:a,b; Jk:a,b;Gm:a,x,g,f,b; Hp:1,2; HLA:16A, 28B.

RESULTS

The observed $\bar{A}$ values with and without the mother for each system examined are displayed in Table 1.

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VII. Biostatistics

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But for one case where both parents and 2 brothers could be studied, the rest, as their progenitors could not be made available, were elucidated through the study of grandparents, uncles, aunts, brothers and sisters, great-uncles and great-grandparents.(3)

In 7 cases the connection with the biologic link was dismissed. In these cases, none of the families keeping the children they alleged to be biologically theirs, and even despite this, would submit to the filiation study.

One abandoned child, after discarding his being a disappeared child and thanks to the efforts of the Abuelas de Plaza de Mayo, could be sent with the mother when the link was confirmed through the study of the genetic markers.

There are Court rulings to analyse 15 children. The abducters of 8 of these children fled once they learned there was a judiciary determination to carry out the studies. These situations, undetached, evidence coarse failures of judiciary and Police custody regarding children suspected of being disappeared ones.

A total of 345 individuals have been studied for the NBGD pertaining to the groups of relatives of 81 disappeared children.

In order to protect their validity, all studies were carried out under the provisions of a Court ruling, or by the Undersecretary of Human Rights.

The support of the international scientific community has been invaluable for the setting out of the National Bank for Genetical Data, especially through the granting of reactives by some centers and also by the contribution of computer programs useful in this field.

We are still in need of such support. For the prosecution of this work a continuous help from the international scientific community is necessary in the following items:

a) Donations of reagents and technical supplies to assure perpetuation of studies, due to Argentina's severely affected economical situation.

b) Assistance to solve complicated scientific and legal problems. e.g. Long time preservation of blood samples with sustained legal validity.

c) Assistance and support for free haemogenetical analysis of relatives living in foreign countries, mainly Europe and United States.

d) Scholarships and scientific assesment.

e) Moral support for those forensic scientists who, by doing this concerned work, receive pressures and threats against their lives.

With this short presentation we want to summarize the contributions of science in general and haemogenetics in particular in favor of the prevalence of Children's Rights, and, among them, specially the fundamental right of identity.

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It was during the last Congress held by the Society for Forensic Haemogenetics that we stressed the fact that most judges and physicians in the Forensic Medical Body lacked the knowledge of the studies on filiation, families looking for their missing children had to face the payment of large sums to obtain analysis and an adequate legal framework get to be set forth, made it tremendously difficult the task of identifying the dissappeared children.(2)

These days, part of this situation has been reverted. For instance and as a consequence of the widespread interest arising from the Court rulings ordering restitution of children after confirmation of their identities through filiation studies, today most of the people of Argentina are aware of the rates of great paternity taht help establish the biological link.

Also, the Parliament has passed two legislations in 1986 and 1987 amending the law on filiation, being the first one, an act incorporating the biological test to determine filiation, thus accepting the biological link.

A Bill regarding a NAtional Bank for Genetic Data on the Next of kin of Disappeared Children was elaborated under the active stimulus of the Abuelas de Plaza de Mayo, together with the Undersecretary of Human Rights, the Secretary for Human and Family Development, both national organizations, the Secretary of the Minor and Family, and the Secretary of Public Health of the Province of Buenos Aires, and the Service of Immunology of Hospital Durand in Buenos Aires. The bill, propelled by President Alfonsín himself, was finally passed last May as Law Nº 23.511.

Such law has ultimately satisfied the long awaited wish of the Grandmothers of Plaza de Mayo for the establishment of practical conditions to enable the identification of their grandchildren however not present, faced with the impossibility of ascertaining when are they to be found. Or, in some other cases, it will be the children themselves, once they have grown up, that will be able to trace their origin through this law, which is likewise built to serve as a weapon against the traffic and trade of children, which is a great problem in our country.

Chief dispositions of this Law are:

1) The creation of the NAtional Bank for Genetic Data which will operate free of charge from the Service of Immunology at Hospital Durand.

2) The determination of the study, in all cases, of the genetic markers of blood groups, of histocompatibility, of seric proteins and erythrocyte enzymes.

3) Keeping blood samples of all next of kin to enable perform the necessary studies arising in the future.

4) Rendering it mandatory for every justice in the nation to perform the pertaining studies of genetic markers on any child with doubtful filiation, to be later checked against those kept at the National Bank for Genetic Data.

5) A set of standards for the procedure and identification of the studies carried out at the NBGD, elsewhere in the country or abroad.

According to life expectancy in Argentina, this NBGD should be operating until, at least, the year 2050.

As from 1984, when the first study was performed, to date, 9 children have been identified through the analysis of genetic markers. In seven cases, the Argentine Courts ruled the plain restitution to the original families, whereas the remaining 2 children are still living with the families who raised them.

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A National Bank for Genetic Data of Disappeared Children in Argentina: Task up to 2050.

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Between the years 1975 and 1983 in Argentina, hundreds of children were kidnapped with their parents or were born during the imprisonment of their mothers in secret detention camps and were then given to persons or families generally connected with the repression body. As demostrated by the investigating Committee, the Democratic Government set forth to investigate human rights violations during the said period, this monstruosity was the product of a deliberate, organic and systematic plan put into execution by the Military Dictatorship in order to produce terror among the people and achieve their objectives. In general, the parents of these children are still missing.(1)

In 1977 the Grandmothers of Plaza de Mayo is created, an organism for human rights, recognized world-wide because of the efforts in trying to return these children to their legitimate parents (families). So far, the Abuelas de Plaza de Mayo (APM) have received reports regarding the disappearances of 255 children, 71 of them kidnapped and 131 having been born in imprisonment. The overall number of disappeared children is estimated to reach 500, all of them on political reasons.

Forty-one (41) of these children have been found as the result of the tireless search their next of kin underwent with the support of the people of Argentina. Four of the children found had died, three of them murdered by the Armed Forces and the other was left to die, abandoned in a hospital.

Eleven (11) children bearing their real names, aware of their background and history and still maintaining a relationship with their real original families, do presently live with the ones who raised them as a result of agreements between the families invloved. Nineteen children have been restituted to their relatives, 7 of them on a Court ruling to do so.

The way in which the restitution is carried out, has been adapted considering the circumstances of each case in particular, counting with the support of a group of persons belonging to different professions formed by doctors, psychologists, lawyers,etc. None of the children suffered psychological descompensation whether at the time of restitution, nor afterwards. In all the cases, the restitution has been a positive and therapeutical event for the child, and all the children enjoy both physical and mental health.

None of these cases proved to call for a return to the situation previous to the restitution. A variety of judges throughout the country have favoured restitution of the dissappeared children to their legitimate families, even of those who were not kept by people connected in any way to the apparatus of repression. The cases of seven children whose original families are known, are being considered by the justice. The expropriators of three of these children fled the moment they learned of the Courts intention to take decissive measures regarding the minors wellbeing.

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In conclusion, populations in China show remarkable heterogeneities from north to south, in sharp contrast to Korean and Japanese populations showed homogeneities, respectively. The center of dispersion of the Gm afb1b3 haplotype characterized southern Mongoloid populations must exist in Gangxi and Yunnan area in the southwest China.

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Chuangs (Guangxi)	0.031	0.005	0.022	0.942	-
Shuis (Sandu)	0.024	0.005	0.019	0.952	-
Koreans (Yanji)	0.491	0.166	0.186	0.157	-

ag=a,z;g,u axg=a,z,x;g,u ab3st=a,z;b0,b3,b5,s,t afblb3= a,f;b0,bl,b3,b4,b5,u

In sharp contrast to Japanese and Korean populations which show homogeneities, respectively, 16 Han nationalities in China indicate clear genocline extending from Haerbin to Guangzhou in which the Gm haplotype frequency of Gm ag changes from 0.471 to 0.168, that of Gm ab3st from 0.117 to 0.033, and that of Gm afblb3 from 0.214 to 0.739, respectively. Even comparing northernmost Beijing with Japanese and Korean populations, highly significant heterogeneities were observed (χ^2=114.58, d.f.=3, p<0.001; χ^2=32.85, d.f.=3, p< 0.001, respectively). In general, Han nationality is clearly divided into two groups of northern and southern; the former has relatively high frequencies of Gm ag and Gm ab3st haplotypes, the latter has low incidences of Gm ag and Gm ab3st and extremely high frequency of Gm afblb3 haplotype.

Of the Minority nationalities Tibetans from Hezhue shows homogeneity with Han from Xian. This may be results of miscellaneous and mingled living of Tibetans and Hans in neighboring surroundings. Tibetans from Lasa and western region in Tibet and Olunchuns from northeasternmost China as well are very characteristic of having high frequencies of Gm ag and Gm ab3st and very low frequency of Gm afblb3 haplotype which characterize northern Mongoloid populations. On the other hand, Miaos, Puyis, Shuis and Chuangs from south China are the most striking ethnic groups to have the highest incidence of Gm afblb3 haplotype which characterizes southern Mongoloid populations. Especially, the incidence of the Gm afblb3 haplotype observed in Chuang and Shuis is the highest value among Mongoloid populations together with Kadazans who are thought to have moved in Borneo 4,000 years B.C. from the southern part of China. Both of Huis from Changji and Uighurs from Wulumuqi in Xinjiang have five Gm haplotypes, Gm fblb3 characteristic of Caucasoids, in addition to the four Gm haplotypes characteristic of Mongoloid populations, which are unique and quite different from other ethnic groups in China. However, Uighurs are in striking contrast to Huis in the frequencies of Gm fblb3 and afblb3 haplotypes. The former have very high frequency of Caucasian haplotype of Gm fblb3 for the latter, on the contrary, Huis have a rather high incidence of Mongoloid haplotype of Gm afblb3 in comparison with Uighurs. They are also different from each other in the frequency of Gm ab3st haplotype. In spite of having lived in a close geographical environment, these ethnic groups are in contrast with each other. It is resonable to say that Huis are basically Mongoloid with some Caucasoid admixture, on the other hand, Uighurs are basically Caucasoid with some Mongoloid admixture.

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the Karakoram and Himalaya ranges on the southwest. China is a multinational country made up of 56 ethnic groups mainly of Han nationality. The characteristic of distribution of multinational ethnic groups, mainly Han over a broad area; for example dispersed over a vast range of land or concentrated in a smaller section of space; has been caused by racial movements, stationing engaged in cultivation, immigration or alterations of dynasties repeatedly over a long history period.

Table 1. Gm haplotype frequencies among various ethnic groups in China

Population	ag	axg	ab3st	afb1b3	fb1b3
Olunchun (NE China)	0.374	0.121	0.440	0.065	-
Tibetans (Lasa)	0.570	0.148	0.213	0.069	-
Tibetans (W Tibet)	0.650	0.159	0.130	0.061	-
Mongols (Wulanhoubu)	0.325	0.209	0.194	0.272	-
Mongols (Inner Mongolia)	0.379	0.190	0.140	0.291	-
Mongols (Huhehote)	0.471	0.203	0.097	0.229	-
Huis (Changji)	0.377	0.108	0.141	0.277	0.097
Uighurs (Wulumuqi)	0.331	0.120	0.113	0.095	0.341
Tibetans (Hezhue)	0.470	0.185	0.128	0.217	-
Han (Haerbin)	0.441	0.210	0.113	0.236	-
Han (Changchun)	0.471	0.219	0.089	0.221	-
Han (Liaoyuan)	0.466	0.237	0.083	0.214	-
Han (Dairen)	0.384	0.266	0.094	0.256	-
Han (Beijing)	0.428	0.214	0.117	0.241	-
Han (Shandong)	0.431	0.190	0.116	0.263	-
Han (Kunsan)	0.376	0.141	0.098	0.385	-
Han (Hefei)	0.416	0.172	0.084	0.328	-
Han (Xian)	0.405	0.183	0.113	0.299	
Han (Hangzhou)	0.350	0.184	0.079	0.387	-
Han (Chengdu)	0.168	0.078	0.048	0.706	-
Han (Changsha)	0.204	0.066	0.054	0.676	-
Han (Gueiyang)	0.226	0.085	0.043	0.646	-
Han (Guangzhou)	0.183	0.054	0.033	0.730	-
Han (Fuzhou)	0.188	0.077	0.043	0.692	-
Han (Taiwan)	0.222	0.087	0.047	0.643	-
Takasagos (Taiwan)	0.194	0.042	0.002	0.762	-
Miaos (Taijing)	0.095	0.015	0.015	0.875	-
Puyis (Duyun)	0.062	0.010	0.014	0.914	-

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Studies on the Human Immunoglobulin Allotypes among Han and Minority Nationalities in China

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INTRODUCTION

Inherited structural differences in human immunoglobulins are referred to as allotypes or genetic markers. So far, genetic markers have been found for the IgG heavy (H) chain (Gm), the IgA H chain (Am), the IgE H chain (Em) and kappa type light (L) chain (Km) common to all classes of immunoglobulins.

Since the discovery of Gm system by Grubb and Laurell(1956), more than twenty allotypes have been described that occur on the H chains of three of the four IgG subclasses, IgG1, IgG2, and IgG3. The Gm system provides genetic markers which are unique in studies of human genetics, particularly in the characterization of different populations and in studies of genetic drift and gene flow determined by the presence of either a unique haplotype in a particular race or by differences in the frequencies of the same haplotypes in a given ethnic group. This paper is one of a series based on studies of genetic markers of immunoglobulins of Mongoloid scattered in the world and neighboring populations.

MATERIALS AND METHODS

Serum samples from a total of 5,048 unrelated individuals from 31 distinct populations in China were tested for G1m(a,z,x, and f), and G3m(b0, b1, b3, b4, b5, s, t, g, u and c3) allotypes. The tests were carried out using previously described methods (Matsumoto and Takatsuki 1968).

RESULTS AND DISCUSSION

China is a vast country with a broad coast bordering the USSR and Mongolian People's Republic on the north and west; it is flanked by Korea on the northeast; the Yellow Sea, East China Sea and South China Sea on the east; Vietnam, Laos and Burma on the south and

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Fig. 1. Map of Mocambique

Fig. 2. Cluster based on gene distance coefficients between 6 southafrican populations: ABO, Rh, MNSs, P, Fy, Jk

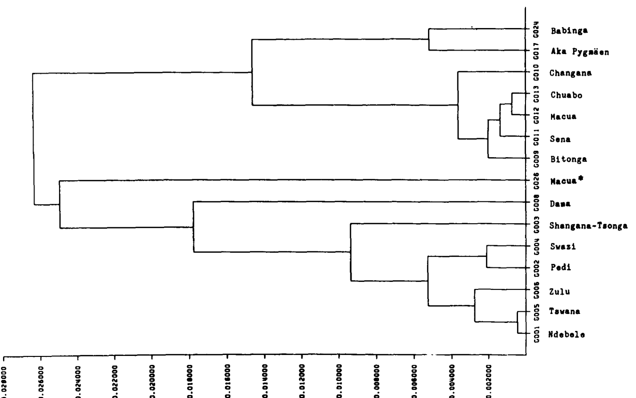

Fig. 3. Cluster based on gene distance coefficients between 15 southafrican populations involving the systems: ABO, Rh, Fy

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Table 2. Red cell enzymes (N=85 for every marker) and serum proteins

System		%	X^2	p	Gene frequen.
AcP	A	3.5	X^2=1.5620	n.s.	A=0.2118
	B	60.0			B=0.7823
	AB	35.5			C=0.0059
	BC	1.2			
PGM_1	1-1	76.5	X^2=0.8970	n.s.	PGM_1^1=0.8706
	2-1	21.2			PGM_1^2=0.1294
	2	2.3			
PGM_2	1-1	94.1	X^2=0.6750	n.s.	PGM_2^1=0.9706
	2-1	5.9			PGM_2^2=0.0294
AK	1	100.0	-	-	AK^1=1.000
	2-1	0.0			
ADA	1	100.0	-	-	ADA^1=1.000
	2-1	0.0			
6-PGD	A	81.2	X^2=1.657	n.s.	6-PGD A=0.9058
	AB	18.8			B=0.0942
	B	0.0			
Gc	1F	64.6			
	1F-1S	9.8	X^2=6.7856	n.s.	1F =0.7866
N=82	1S	2.4	df=3		1S =0.0793
	2-1F	17.1			2 =0.1219
	2-1S	1.2			2A3=0.0122
	2	2.4			
	1F-2A3	1.2			
	2 -2A3	1.2			
Tf	C1	69.5			
	C1-C2	11.0	X^2= 1.7868	n.s.	C1=0.8232
N=82	C2	4.9	df=3		C2=0.1036
	C1-D1	14.6			D1=0.0732
Pi	M_1	81.2	X^2=1.975	n.s.	M_1=0.8913
	M_1M_2	2.9	df=3		M_2=0.0217
	M_1M_3	13.0			M_3=0.0870
	M_2M_3	1.5			
	M_3	1.5			

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Table 1. Distribution of ABO, MNSs, Rhesus, Fy, Jk, Lu, P, Kell phenotype and gene frequencies of the Macua

System		%	X^2	p	Gene frequen.
ABO	A	22.9	X^2=0.140	n.s.	p=0.1925
♂ N=609	B	16.8			q=0.1148
	AB	4.9			r=0.6959
	O	48.4			
♀ N=320	A	35.3	X^2=0.165	n.s.	p=0.2356
	B	17.5			q=0.1269
	AB	6.3			r=0.6379
	O	40.4			
Rhesus	+	98.4		n.s.	Do=0.83
♂ N=609	-	1.6			do=0.17
♀ N=302	+	98.5		n.s.	Do=0.83
	-	1.5			do=0.17
N=109	CCDee	1.8	X^2(Rh/rh)=5.68	p= .05	cde=0.3831
	CcDEE	0.9			cdE=0.0120
	CcDEe	2.8			Cde=0.0239
Rh^-	CcDee	21.1			cDe=0.4296
	ccDEE	1.8			CDe=0.1163
	ccDEe	2.8			cDE=0.1622
	ccDee	51.4			C=0.1514
	ccddee	14.7	X^2=0.1389	n.s.	c=0.8486
Rh^+	Ccddee	1.8			E=0.0596
	ccddEe	0.9	X^2=19.14	$p<0.001$	e=0.9404
MNSs	MMSS	6.4	X^2=11.52	$p<0.001$	MS=0.2166
N=109	MMSs	14.7			Ms=0.3430
	MMss	14.7			NS=0.0292
	MNSS	6.4			Ns=0.4112
	MNSs	12.8			M=0.5596
	MNss	21.1			N=0.4404
	NNSS	2.8			S=0.2936
	NNss	21.1			s=0.7064
P_1	P_1+	96.3		n.s.	P_1=0.81
N=109	P_1-	3.7			
Kell	K^-	100.0		-	-
N=109					
Fy	a-b+	2.8	X^2=0.0203	n.s.	Fy^o=0.9869
N=109	a-b-	97.2			Fy^a=0.0138
Lu	a+b+	23.8	X^2=0.3873	n.s.	Lu^a=0.1190
N=109	a-b+	76.2			Lu^b=0.8810

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populations. Further frequencies of other phenotypes like A1B (8.3%), NNss (21.1%), Jka+b+ (39.54%) and Lua+b+ (23.8%) found in our sample, differ also from the other populations. No definite conclusion could be reached from this observation in this small population.

The serum protein phenotypes 1F (Gc, 64.6%), M1 (Pi, 81.2%) and C1 (Tf, 69.5%) were the most common alleles in this group. Rare phenotypes were 2-1S,1F-2A3,and 2-2A3 in Gc,M3,M1M2,and M2M3 in Pi.One rare Gc variant 2A3 was found in 2 individuals;however,the Gc variant 1A1 and Tf- C3 in our sample were not observed.
The gene frequencies observed in our sample for the red cell enzymes were within the range expected for South African populations studied until now. The phenotypes Ak 2-1/2, ADA 2-1/2, PGM2 2-2, AcP AC/C/RA and 6-PGD B were absent in our sample.

A comparison of our results with the previously published in the literature show that the genetic distance between the two Macua populations (ours and from Da Cunha et al. 1970) is greater than the one observed between the Central Bantu and the Shona/Thonga coast populations. The cluster analysis (fig. 2 and 3) shows, that our Macua group is closer related to the Sena and Shona/ Thonga coast populations, than to the other Macua and Bantu populations. Somewhat, greater heterogenity is observed between our Macua group and other Bantu groups with respect to allelic frequencies for Rh and MNSs. The presence of some rare allele of serum proteins in Macua Negroids gives new anthropological and ecological perspectives of great interest. Further investigations on the polymorphism of the serum proteins and enzymes are needed to give a wider horizon to the biological anthropology of South Africa. Microevolutionary forces like genetic drift combined with founder effect and endogamy are the probable causes for the observed variation of allelic frequencies in the populations studied. Considering the great ethnic heterogenity of the mentioned groups and the diversity of their habitat, the south african populations of Mocambique are largely heterogenous.

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Blood groups, serum proteins and red cell isoenzymes - a population genetic study on South Africa/Mocambique.

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This study is a part of a general survey aimed at investigating the genetic variability of serological and anthropological characters and to measure the genetic relationship between indigenous groups in South Africa. The blood samples were obtained from individuals living in the districts Cabo Delgado around the village Pemba (eastern pacific coast). Inspite of sporadic marriage contacts with the neighbouring groups, the people of Pemba (all Macua of Bantu origin) practice endogamy (contracting marriages only within their own group); this region has not yet been affected by social changes in the family structure and marriage system. These people share a common dialect and a common ecological zone. Thus, they can be regarded as an homogenous group with respect to linguistical, social, economic and cultural factors (fig. 1).

MATERIAL AND METHODS

109 blood samples were taken from unrelated donors of tribe Macua living around Pemba, and stored at 4°C, until their arrival in West Germany. For ABO and Rh (D factor only) 929 blood data from Blood Bank-Pemba were used. 85 samples (serum and cloths) were stored at -22°C.

We have typed: ABO, CcDEe, MNSs, Fy, Jk, Kk, Lu and P1 (blood groups); Gc Tf, Pi (IEF) (serum proteins); AcP, Ak, ADA, 6-PGD, PGM1 and PGM2 (erythrocyte enzymes); (electrophoresis on celulose acetate foils gel = CAFG). X^2-Test was used to examine the heterogenity and the genetic distance analysis (Edwards and Cavalli-Sforza, 1972) was carried out to compare the data with those of other studies of South African populations.

RESULTS AND DISCUSSION

Table 1 and 2 show the distribution of blood groups, serum proteins, red cell enzymes and their gene frequencies, respectively. The distribution of observed and expected phenotype frequencies are, with exception of the Rhesus (D and E locus) and MNSs systems, in good agreement with the expected Hardy-Weinberg proportion. The frequency of the Rhesus phenotypes CcDee (22.9%), ccddee (14.7%) found in our sample were also relatively high and differ significantly from the ours observed in other South African

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TABLE 3 GLO1 segregation analysis. Family material from SW Germany.

mating type male/female	Nr. fam.	Nr. child.	Phenotypes of the children 1	2-1	2	X2
1 x 2-1	29	82	37	45		.780
2-1 x 1	34	102	45	57		1.412
2-1 x 2-1	81	240	51	133	56	3.025
2-1 x 2	63	169		95	74	2.609
2 x 2-1	56	190		113	77	6.821

TABLE 4 PGP segregation analysis. Family material from SW Germany and NW Portugal.

mating type male x female	Nr. fam.	Nr. child.	Phenotypes of the children 1	2-1	X2
1 x 2-1	22	46	18	28	1.562
2-1 x 1	24	70	46	24	6.914
TOTAL	46	116	64	52	1.241

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TABLE 1 Gene frequencies and sample sizes used for segregation analysis. G and P stand respectively for SW Germany and NW Portugal.

System	Sample	Nr. fam.	Nr. child.	Frequencies of the alleles(%) 1	2	3	5	V
ALAD	G	222	629	92	8			
	P	133	263	89	11			
AMY2	G	258	784	97	3			
	P	108	230	94.2	5.6	0.2		
ESD	G	176	494	86.2	12.5		1.3	
	P	140	486	86.1	13.0		0.7	0.2
GLO1	G	210	697	43	57			
	P	109	242	44	56			
HP	G	91	268	40	60			
	P	110	247	39	61			
ORM	G	159	479	61	39			
	P	35	47	61	39			
PGP	G	184	486	87	10	3		
	P	113	242	92	5	3		

TABLE 2 AMY2 segregation analysis. Family material from SW Germany and NW Portugal.

mating type male	x female	Nr. fam.	Nr. child.	Phenotypes of the children 1	2-1	X2
1	2-1	19	67	46	21	9.33
2-1	1	30	81	49	32	3.57
TOTAL		49	148	95	53	11.92

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GLO1

Two mother/child exclusions were found, one in each sample (estimated GLO1*O frequencies: 0.001 in SW Germany and 0.002 in NW Portugal).

Concerning the segregation analysis, in the Portuguese material the offspring of 2-1x2-1 mating type show a significant deviation from expected values (X2=6.55, 2 d.f.); in the German sample a general trend to homozygote excess is also observed (Table 3). On the other hand, in the Portuguese sample the offspring of mating type: father 2-1 x mother 1 show a significant sex/phenotype association.

HP

A mother/child exclusion was found in the Portuguese sample (estimated HP*O frequency: 0.002).

Apart from that, the observed segregation results agree well with expected ones.

ORM

No mother/child exclusions were found.

The observed segregation results agree well with expected ones, but in the Portugese sample a significant deviation was found in mating type 1x2-1 (16 vs. 6 children respectively 1 and 2-1). The small sample size prevents us from giving relevance to this finding.

PGP

No mother/child exclusions were found.

Concerning segregation analysis, a significant distortion was found in both samples for mating type: father 2-1 x mother 1, but not in its reciprocal (Table 4).

CONCLUSIONS

The results here reported confirm the need for regular formal genetics researches on all genetic systems used in paternity expertises, including those for which the formal genetic model is considered to be established.
Furthermore, the fact that in some of the markers here studied the detected segregation disturbancies where present in independent samplings geographically rather distant, seems to substantiate the conclusion that at least for these markers the deviations are not due to mere chance and deserve a further investigation.

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Formal genetics studies on ALAD, AMY2, ESD, GLO1, HP, ORM and PGP polymorphisms

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The genetic polymorphisms of ALAD (aminolevulinate dehydratase, E.C.4.2.1.24), AMY2 (pancreatic amylase, E.C.3.2.1.1), ESD (esterase D, E.C.3.1.1.1), GLO1 (glyoxalase I, E.C.4.4.1.5), HP (haptoglobin), ORM (orosomucoid) and PGP (phosphoglycollate phosphatase, E.C.3.1.3.18) are currently used in paternity expertises.

The purpose of this work is to perform a revision of the formal genetics of these systems, using family material from SW Germany and NW Portugal.

MATERIAL AND METHODS

Phenotyping methods are described in previous publications of the research groups involved.

RESULTS AND DISCUSSION

In Table 1 we present sample data and calculated gene frequencies for each marker in SW Germany and NW Portugal.

ALAD

No mother/child exclusions were found.

The observed segregation results agree well with expected ones.

AMY2

Two mother/child exclusions were found, one in each sample (estimated AMY2*0 frequencies: 0.001 in SW Germany and 0.002 in NW Portugal).

On the other hand, the segregation in the offspring of mating type 1x2-1 is clearly distorted (Table 2).

ESD

No mother/child exclusions were found.

The observed segregation results agree well with expected ones, if the sex of children is not taken into account. However, a significant sex/phenotype association was found in both samples when analysing mating types 1x2-1 and its reciprocal.

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the Hardy-Weinberg equilibrium.
Furthermore they showed no significant difference compared with data published by Simeoni and Grüner(1983) for the North of Germany and the distribution in the Netherlands(Klasen et al.)
For transferrin 12 different phenotypes(C1-1,C2-1,C3-1,C10-1, C1-B,C1-D,C2-2,C2-B,C3-2,C3-3,B-B,B-D)and the gene products C1, C2, C3, C10, B and D could be defined.
The number of phenotypes observed corresponds well to the figure expected under Hardy-Weinberg equilibrium.
The allele frequencies in the group of typed unrelated adults are in good agreement with the data published by Rand,Kofahl and Brinkmann (Münster 1985) and Weidinger (München 1980).
For parentage problems and clinical purposes the described Pi- -and Tf-subtyping in Immobiline gel is a fast and simple method, giving reliable results.

Alpha-1-Antitrypsin

N	M1-1	M2-1	M3-1	M1-var	M2-2	M2-var	M3-2	M3-3	M3-var	
500	268	97	66	26	15	5	16	5	2	obs.
500	263	107	68	25	11	5	14	4	3	exp.

$X^2 = 3.44$ df= 6 p= 0.75

Gene frequencies: M1=0.725 M2=0.148 M3=0.094 S=0.021
Z=0.007 F=0.005

Transferrin

N	C1-1	C2-1	C3-1	C10-1	C1-var	C2-var +var	C2-2	C3-2	C3-3	
500	302	129	37	1	9	3	11	7	1	obs.
500	304	124	37	1	11	2	13	7	1	exp.

$X^2 = 1.384$ df= 5 p= 0.92

Gene frequencies: C1=0.780 C2=0.159 C3=0.046 C10=0.001
Var=0.014

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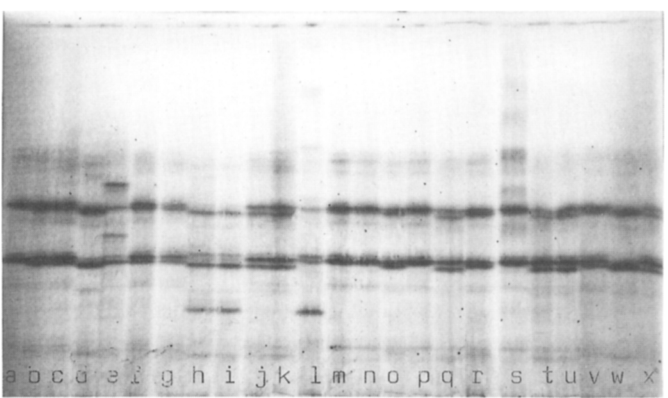
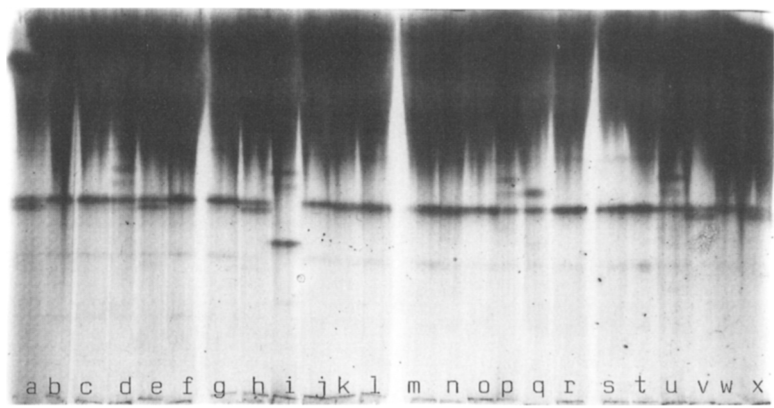
Fixation,staining,destaining and preservation according to LKB-Instructions(1804)



Alpha-1-Antitrypsin phenotypes in figure 1:
a:M1-1,b:M3-1,c:M3-1,d:M2-2,e:M3-F,f:M3-1,g:M3-1,h:M2-S,i:M2-S,
j:M2-1,k:M2-1,l:M1-S,m:M1-1,n:M1-1,o:M3-1,p:M1-1,q:M2-1,r:M3-1,
s:M1-1,t:M2-1,u:M2-1,v:M3-1,w:M3-2,x:M2-1.



Transferrin phenotypes in figure 2:
a:C2-1,b:C1-1,c:C1-1,d:C1-B,e:C2-1,f:C1-1,g:C1-1,h:C2-1,i:B-D,
j:C1-1,k:C3-1,l:C3-1,m:C3-1,n:C3-1,o:C1-1,p:C1-B,q:C1-B,r:C1-1,
s:C1-1,t:C1-1,u:C1-B,v:C2-1,w:C1-1,x:C2-1

Results:
For alpha-1-antitrypsin the gene products M1, M2, M3, F, S and Z could be defined.
Typing a group of 500 healthy,unrelated blood donors 13 different phenotypes(M1-1,M2-1,M3-1,M1-F,M1-S,M1-Z,M2-2,M2-F, M2-S,M3-2,M3-3,M3-F,M3-S)were found.
The observed values agreed well with the expected ones,assuming

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POLYMORPHISMS OF ALPHA-1-ANTITRYPSIN AND TRANSFERRIN IN NORTH-WEST GERMANY

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Aims of the study:
- Investigation of the applicability of Immobiline dry plates for the determination of alpha-1-antitrypsin(Pi) and transferrin(Tf) polymorphisms.
  The system to define alpha-1-antitrypsin gene products was also set up in view of the diagnosis and prevention of premature emphysema and progressive liver disease.
- Population genetics of alpha-1-antitrypsin and transferrin in North-West Germany.

Alpha-1-Antitrypsin
Method: Electrofocusing in Immobiline gel, pH-range 4.2-4.9 (LKB, Bromma, Sweden).
1.Rehydration: Immobiline dry plate 16 hours in the reswelling cassette with 25% glycerol.
2.Sample application: 5 µl serum were applied to filter paper pieces, laid on the gel surface about 1 cm from the cathode, by using a multiple microlitre syringe (6x50 µl Hamilton).
3. Electrode solutions: Anode and cathode: distilled water
4. Running conditions: Electrical settings: 5000 V, 2 mA, 5 W
Cooling plate: 8° C
Running time: 1 hour with sample application pieces, 6 hours without.
No prefocusing

Transferrin
Method: Electrofocusing in Immobiline gel, pH-range 5.0-6.0 (LKB, Bromma, Sweden)
1.Sample preparation: 5 µl serum and 25 µl 0.25% ferric ammonium sulphate over night at 4°C.
2.Rehydration: Immobiline dry plate 2 hours in the reswelling cassette with 25% glycerol.
3.Sample application: 5 µl of the prepared serum were applied to filter paper pieces, laid on the gel surface about 1 cm from the cathode, by using a multiple microlitre syringe (6x50 µl Hamilton)
4.Electrode solutions: Anode and cathode: distilled water
5.Running conditions: Electrical settings: 3000 V, 4 mA, 12 W
Cooling plate: 8° C
Running time: 1 hour with sample application pieces, 3.5 hours without.
No prefocusing

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three appear to be associated with a severe degree of deficiency (< 5%), the previously described G6PD Cagliari, G6PD Sassari, and G6PD Mediterranean variants (Testa et al. 1980; Fenu et al. 1982). The remaining twelve are associated with a mild to moderate level of G6PD deficiency.

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Table 1. Relative frequency of severe and moderate G6PD deficency in various Italian populations.

Origin	Mild	Severe	Total
Sardinia general	0.010	0.120	0.130
Bari	-	0.002	0.002
Napoli	0.015	0.007	0.022
Matera	0.008	0.020	0.028
Southern Italy general	0.015	0.007	0.022
Agrigento	0.021	-	0.021
Caltanissetta	0.007	0.021	0.028
Catania	0.028	0.006	0.034
Messina	0.015	0.005	0.020
Palermo	0.010	0.005	0.015
Ragusa	0.047	0.016	0.063
Siracusa	0.034	0.011	0.045
Sicily general	0.018	0.007	0.025

Genetic Basis of Deficiency

On the basis of electrophoretic analysis and enzyme activity assay on red blood cells lysates and of biochemical analysis on partially purified enzyme preparations it has been possible to identify the number and type of protein variants which account for G6PD deficiency in various populations of Southern Italy (Table 2).

Table 2. Minimum number of common genetic variants in Italian populations and associated degree of deficiency.

Origin	Total	Number of variants associated with Mild enzyme deficiency	Number of variants associated with Severe enzyme deficiency
Sardinia	4	1	3
Sicily	5	4	1
Southern Italy	11	10	1

For each variant the familiar segregation has been demonstrated. On the basis of this analysis we have established that the minimum number of common Gd genetic variants segregating in the Italian population is fifteen, eleven of which had been already described (Testa et al. 1980; Fenu et al. 1982; Colonna Romano et al. 1985). The four new variants have different than normal electrophoretic mobility (namely 90, 93, and 110% of normal), with two variants showing the same mobility (90%) but easily distinguishable on the basis of enzyme activity ($< 15\%$ and $> 25\%$ respectively). Moreover these variants show normal kinetic parameters. Among the fifteen variants only

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the Southern and Eastern coasts of the island.

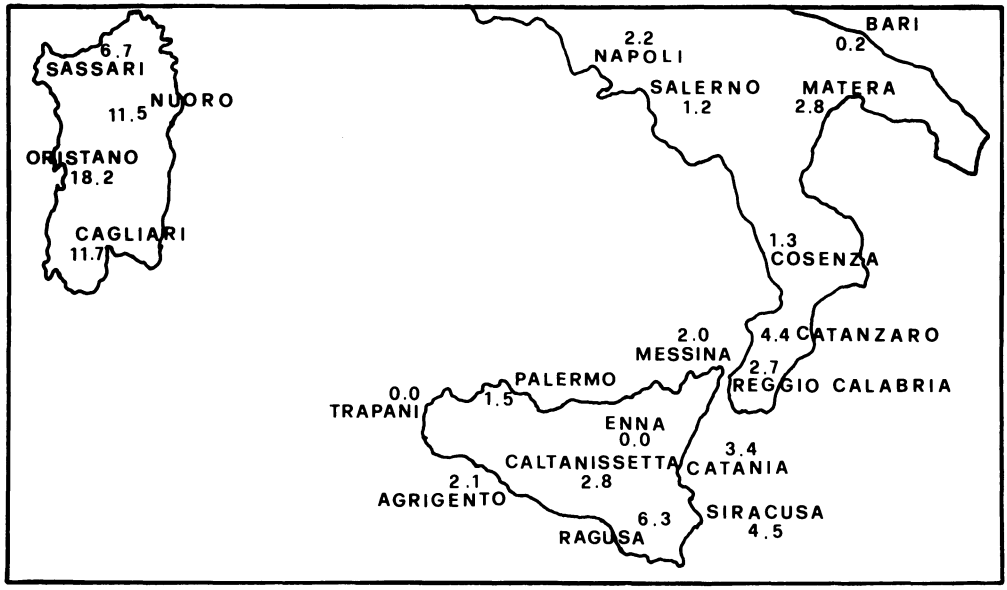

Fig. 1. Distribution of the frequency of G6PD deficiency in Southern Italy.

In the Matera province, the frequency of G6PD deficiency appears to be inversely related to the distance of each examined town from the Ionian coast, the greater the distance the lower the frequency. In both cases the pattern of variation of G6PD(-) frequency appears to be related to the population origin and migrational pattern and to the altitude, which in turn is known to be correlated with past malarial endemia (Siniscalco et al. 1966).

Type of Deficiency

The analysis of the enzyme activity level evidences a further element of variation within and between populations. At least two types of deficiency can be easily recognized in each population. A class of subjects is characterized by an extremely low level of G6PD activity in red blood cells ($< 5\%$) while others show an activity level ranging from 15 to 50% of normal G6PD B activity.

The relative proportions of these two classes of individuals vary considerably according to the population examined. In Sardinia as far as 92% of deficient subjects are included in the severe deficiency class. However, in Napoli severe deficiency accounts for only 30% of the total frequency (Table 1).

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Distribution of Genetically Determined Deficient Variants of Glucose-6-Phosphate Dehydrogenase (G6PD) in Southern Italy.

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INTRODUCTION

The genetic locus coding for G6PD (*Gd*) is located at the tip of the long arm (q28) of the human X chromosome leading to the typical "sex linked" pattern of inheritance of G6PD genetic variants. Thus variants are fully expressed, and therefore easily recognized, in hemizygous male subjects. *Gd* locus is highly polymorphic in human populations living in countries lying between 45° N and 35° S Latitude. Genetic polymorphism is mostly due to the occurrence within a given population of one or more variants associated with a reduced level of enzyme activity in red blood cells, together with the common G6PD B form (Luzzatto and Battistuzzi 1985).

MATERIALS AND METHODS

Blood specimens were collected by venepuncture from 15,000 unrelated male donors, representative of the various populations of Southern Italy. G6PD phenotype was determined on red blood cells lysates according to Betke et al. (1967) on cellulose acetate strips. Subjects showing a variant phenotype were further characterized in terms of enzyme activity level, electrophoretic mobility, and biochemical characteristics (Modiano et al. 1979).

RESULTS AND DISCUSSION

Distribution of G6PD Deficiency

The highest proportion of G6PD deficient subjects was found in the island of Sardinia, where 13% of the male population carry a G6PD variant with reduced enzyme activity (Fig. 1). A much lower frequency of G6PD deficiency is found in the Sicilian and peninsular population (2.5% and 2.2%). A detailed analysis of the distribution of G6PD deficiency within each area showed a considerable amount of variation. In Sardinia, the highest frequency is found in the province of Oristano (18%) while only approximately one third of this frequency (6.7%) is found in the nearby area of Sassari. An even more striking example of geographic variation of G6PD(-) frequency is shown by the populations of Sicily and of the Matera area. In the former case, the highest frequency is found in the "Val di Noto", the southernmost corner of the island, specifically in Ragusa (6.3%) and Siracusa (4.5%). From this level, the frequency of G6PD deficiency smoothly decreases along

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Table 1: Phenotype distribution and gene frequencies in a sample from Tuscany (Italy)

Phenotype	Observed n	Observed %	Expected n	Expected %	Gene Frequencies
1	181	51.71	182.16	52.05	A2HS*1 = .7214 ± .0169
2-1	142	40.57	139.96	39.99	A2HS*2 = .2771 ± .0169
2	26	7.43	26.88	7.68	A2HS*Rare = .0014 ± .0014
1-Rare °	1	0.29	0.72	0.21	
2-Rare °	0	0.00	0.28	0.08	
Rare °	0	0.00	0.00	0.00	
Total	350	100.00	350.00	100.00	

chi square: 0.0655, 1 d.f., P> 0.20
°(not included in the chi square test)

Table 2: Geographic distribution of A2HS gene frequencies

Population	n	A2HS*1	A2HS*2	A2HS*Rare	References	
Norway	52	.6000	.3900	--	Olaisen et al.	1981
Canada (Toronto)	215	.6419	.3535	.0046	Cox et al.	1986
Germany (Hessen)	197	.6550	.3400	.0050	Tarkkala Mendner	1986
Germany (Munchen)	166	.6540	.3220	.0240	Weidinger et al.	1984
Germany (South)	344	.6642	.3208	.0150	Weidinger	1986
Italy (Tuscany)	350	.7214	.2771	.0014	Present Study	
Japan (Yamagata)	2050	.7356	.2639	.0005	Umetsu et al.	1984
Japan (Izumo)	300	.7233	.2767	--	Yuasa et al.	1985
Japan (Yamaguchi)	400	.7325	.2675	--	Yuasa et al.	1985
Japan (Okinawa)	397	.7670	.2065	.0264	Yuasa et al.	1985
Carribean (Black)	71	.6901	.2606	.0493	Cox et al.	1986

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temperature gel was stored overnight at 4 °C.
Sample application: samples of 8 μl were applied 1.5 cm from the cathode using applicator strips from Serva.
Electrode solutions: anode : 0.025 M aspartic acid + 0.025 M glutamic acid; catode: 0.1 M NaOH.
Isoelectric focusing (without prefocusing): in a Multiphor chamber (LKB 2117) at a cooling temperature of 8 °C for 3 h: 2000 V, 20 mA, 8 W for 1 h; 2000 V, 20 mA, 18 W for 2 h.
Immunoprinting: after isoelectric focusing, on gel surface were applied cellulose acetate strips (from Gelman Sci., Inc. Ann Arbor - Michigan USA) soaked in a saline diluted 1/2 anti human-2-HS-Glycoprotein antiserum (from Behering) for 3′ at room temperature. Strips were removed and washed twice in saline for 15′, stained for 5′ with nigrosine and destained for 10′ by an acetic acid 5 % solution.

RESULTS AND DISCUSSION

Distribution of the observed phenotypes is given in table 1. The good agreement between the observed and expected phenotypes, assuming Hardy-Weinberg equilibrium condition, is self- evident. The estimated allele frequencies from our population sample are: A2HS*1 = 0.7214, A2HS*2 = 0.2771, A2HS*Rare = 0.0014.
The product of the rare allele we detected in our sample is still to be classified.
Table 2 summarize the hitherto reported A2HS allele frequencies in various population. The estimated frequency of A2HS*1 allele in Central Italy appears higher than in both North European and North American countries. It seems closer to the A2HS*1 gene frequency in Oriental populations.
A2HS-glycoprotein polymorphism is suitable for being applied to the investigation of disputed paternity. In Italian population the theoretical chances of exclusion is about 16.2 % (I class exclusion: 8.2 %; II class exclusion: 8.0 %).

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ALPHA-2-HS-GLYCOPROTEIN POLYMORPHISM IN TUSCANY (ITALY)

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INTRODUCTION

The genetic polymorphysm of A2HS glycoprotein was described for the first time in 1977 by Anderson and Anderson. Using two-dimensional electrophoresis they detected three common phenotypes controlled by two codominant autosomic alleles, which they named L and N.
The study of A2HS system was remarkably improved in 1983 when isoelectric focusing followed by immunofixation was introduced independently by Cox et al. and Umetsu et al.
By means of that technique the products of the two common alleles, A2HS*1 (= L) and A2HS*2 (= N) may be recognized in all the examined populations. In Caucasian population also allele A2HS*3 (Cox et al., 1983), which is polymorphic in some geographic areas, allele A2HS*4 (Weidinger et al., 1984) and A2HS*10 (Weidinger et al., 1986) have been described. Several alleles have detected in the Orientals, including A2HS*5 (Umetsu 1984, b), which is polymorphic in Southern Japan.
A2HS locus is placed in chromosome 3 (Cox et al., 1985).
The genetic plymorphism of A2HS glycoprotein had not been investigated in Italy until now. In this paper the distribution of A2HS phenotypes in a sample of the population of Tuscany (Central Italy) has been reported.

MATERIALS AND METHODS

Sample collection: 350 samples of serum obtained from unrelated blood donors of the Hospital of Pisa have been investigated. Sera have been tested within three days from the drawing of blood. Until use sera were stored at -20 °C.
Phenotypes have been recognized using isoelectric focusing in polyacrylamide gels followed by immunofixation, according to the technique suggested by Weidinger (1986) with minor modifications.
Gel casting: isoelectric focusing was carried out with flat bed gels (250 x 115 x 0.5 mm) using glass plates treated with methacryloxypropyltrimethoxysilane; 9.0 ml solution of acrylamide (19 % w/v) + bis-acrylamide (0.6 % w/v), 1.0 ml Pharmalyte pH 4.2-4.9, 0.2 ml Pharmalyte pH 4.5-5.4, 2.4 ml Glycerol (87 %), 5.5 ml distilled water, 30 mg ACES (Serva) were mixed in a flask and deaerated for 5 min. After addition of 8 µl TEMED and 160 µl ammonium persulfate solution (3% in distilled water), gels were polymerized for 30 min. After 1 h at room

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Table 6: Pi allele frequencies distribution in Italy

ITALY	n	Pi*M1	Pi*M2	Pi*M3	Pi*S	Pi*Z	Pi*Rare	Ref
North	1973	.7082	.1828	.0697	.0243	.0075	.0042	2,4
Center	3253	.6800	.1791	.0999	.0300	.0054	.0054	2,4,9
Continental	5226	.6907	.1805	.0885	.0278	.0062	.0049	
Nuoro	205	.6530	.3100°		.0300	.0070	.0000	7
Cagliari	316	.6110	.3300°		.0550	.0040	.0000	7
Cagliari	500	.6140	.2100	.1320	.0420	.0010	.0010	
Sardinia	1021	.6209	.3319°		.0436	.0031	.0005	

(°) Pi*M2 + Pi*M3 + Pi*M4

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Table 4: Gc allele frequencies distribution in Italy

ITALY	n	Gc*1F	Gc*1S	Gc*2	Gc*Rare	Ref.
North	2297	.1508	.5707	.2773	.0011	2,5
Center	3451	.1407	.5952	.2634	.0007	2,8
South	398	.1332	.5967	.2701	.0000	2
Continental	6146	.1440	.5862	.2690	.0008	
Nuoro	205	.1660	.5940	.2400	.0000	7
Nuoro	343	.1790	.5350	.2770	.0090	2
Cagliari	316	.1870	.5540	.2590	.0000	7
Cagliari	500	.1940	.5410	.2590	.0060	
Sardinia	1364	.1844	.5506	.2606	.0044	

Table 5: Tf allele frequencies distribution in Italy

ITALY	n	Tf*C1	Tf*C2	Tf*C3	Tf*Rare	Ref.
North	1749	.7810	.1667	.0492	.0031	2,5
Center	2691	.7660	.1735	.0557	.0048	2
South	1007	.7631	.1773	.0546	.0050	2
Continental	5447	.7703	.1719	.0534	.0043	
Sardinia (Cagliari)	500	.7510	.2060	.0330	.0100	

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Table 2: Tf phenotype distribution and gene frequencies in a sample from Cagliari (Sardinia)

Phenotype	Observed n	Observed %	Expected n	Expected %	Gene Frequencies
C1	290	58.00	282.00	56.40	Tf*C1 = 0.751
C1-2	146	29.20	154.70	30.94	Tf*C2 = 0.206
C1-3	20	4.00	24.78	4.96	Tf*C3 = 0.033
C2	23	4.60	21.21	4.24	Tf*Rare = 0.010
C2-C3	10	2.00	6.79	1.36	
C3	1	0.20	0.54	0.11	
C1-Rare	5	1.00	7.51	1.50	
C2-Rare	4	0.80	2.06	0.41	
C3-Rare	1	0.20	0.33	0.07	
Rare	0	0.00	0.05	0.01	
Total	500	100.00	500.00	100.00	

chi square : 7.7550, 6 d.f., P > 0.20

Table 3: Pi phenotype distribution and gene frequencies in a sample from Cagliari (Sardinia)

Phenotype	Observed n	Observed %	Expected n	Expected %	Gene Frequencies
M1	195	39.00	188.50	37.70	Pi*M1 = 0.614
M1-2	124	24.80	128.94	25.79	Pi*M2 = 0.210
M1-3	77	15.40	81.05	16.21	Pi*M3 = 0.132
M2	23	4.60	22.05	4.41	Pi*S = 0.042
M2-3	28	5.60	27.72	5.54	Pi*Rare = 0.002
M3	10	2.00	8.71	1.74	
M1-S	22	4.40	25.79	5.16	
M2-S	11	2.20	8.82	1.76	
M3-S	7	1.40	5.54	1.11	
S	1	0.20	0.88	0.18	
M1-Rare	1	0.20	1.23	0.25	
M2-Rare	1	0.20	0.42	0.08	
M3-Rare	0	0.00	0.26	0.05	
S- Rare	0	0.00	0.08	0.02	
Rare	0	0.00	0.00	0.00	
Total	500	100.00	500.00	100.00	

chi square : 3.5366, 10 d.f., P > 20

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distribution of some genetic polymorphisms in Italy" edited in 1982 by the G.E.F.I. (Gruppo degli Ematologi Forensi Italiani: see Piazza et al. 1982).
Table 4 shows that in Sardinia Gc*1F has a higher frequency than in any other part of continental Italy, though the examination of the single samples shows a slight overlapping of the continental upper values with lower insular ones (Giari et al., in press).
The same observation can be made about the allele Tf*C2 frequency (see table 5), but in this case the disparity is less marked.
From table 6 appears that the average frequency of Pi*M1 in Sardinia is about 7% less than in continental Italy. Also for this polymorphism we have noted an overlapping which has the same characteristics of that concerning Gc*1F.
In our opinion the present data confirm that is convenient to keep the distinction between the average allelic frequencies of Sardinia and those of the rest of Italy, to calculate the probability of paternity.

TABLES

Table 1: Gc phenotype distribution and gene frequencies in a sample from Cagliari (Sardinia)

Phenotype	Observed		Expected		Gene Frequencies	
	n	%	n	%		
1S	143	28.60	146.34	29.27	Gc*1S	= 0.541
1F-1S	108	21.60	104.95	20.99	Gc*1F	= 0.194
1F	16	3.20	18.82	3.77	Gc*2	= 0.259
2	32	6.40	33.54	6.71	Gc*Rare	= 0.006
2-1S	142	28.40	140.12	28.02		
2-1F	53	10.60	50.25	10.05		
1S-Rare	5	1.00	3.25	0.65		
1F-Rare	1	0.20	1.16	0.23		
2- Rare	0	0.00	1.55	0.31		
Rare	0	0.00	0.02	0.00		
Total	500	100.00	500.00	100.00		

chi square : 3.376, 6 d.f., $P > 0.20$

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DISTRIBUTION OF Gc, Pi AND Tf SUBTYPES IN SARDINIA (ITALY)

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INTRODUCTION

The differences of values of allelic frequencies of Sardinians compared to those of the rest of Italy, which have been demonstrated for several polymorphisms, are due to the peculiar ethno-geographic situation of the island. The studies of the distribution of the genetic polymorphisms in Sardinia is interesting not only for the geneticist of population or the anthropologist, but it is useful also for the investigations about disputed paternity. This is because the use of the continental average gene frequencies, instead of the insular ones, may cause disparities, sometimes even significant ones, in the calculation of probability of paternity.
This study aims to contribute to the knowledge of the distribution of the allelic frequencies of the Gc, Pi and Tf subtypes in Sardinia.

MATERIALS AND METHODS

Sera were collected from 500 unrelated blood donors originating from Cagliari, Sardinia (all their parents and grandparents were born in Cagliari Province).
Gc, Pi and Tf typing was performed as elsewhere described (Bargagna et al., 1983).

RESULTS AND DISCUSSION

The distribution of phenotypes and gene frequencies estimated by our sample for the Gc, Tf and Pi polymorphism are reported in table 1, 2 and 3 respectively. In table 3, Pi*Rare includes Pi*Z and the other uncommon variants Pi: the gene frequencies are Pi*Z = 0.001, Pi*Var. = 0.001, respectively. The observed and expected values assuming a Hardy-Weinberg equilibrium were in good agreement.
Tables 4, 5 and 6 compare the Sardinian allelic frequencies to the continental Italy average ones. The majority of data from which we have obtained the average allelic frequencies comes from the papers gathered for the preparation of the second edition of the monograph "The

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TABLE 3. The distributio of Bf phenotypes and gene frequencies in the Veneto population.

PHENOTYPES	OBSERVED n	OBSERVED %	EXPECTED n	EXPECTED %
S	324	54.72973	324.05653	54.73928
FS	199	33.61486	199.76163	33.74352
F	31	5.230649	30.78630	5.20039
S-S0.7	13	2.19595	12.67917	2.14175
S-F1	16	2.70270	15.64012	2.64191
F-S0.7	4	0.67568	3.97813	0.67198
F-F1	5	0.84459	4.78975	0.80908
S0.7	-	-	0.12207	0.02062
F1	-	-	0.18630	0.03147
	592	100.00000	592.00000	100.00000

Bf^{S}=0.73986, Bf^{F}=0.22802, $Bf^{S0.7}$=0.01436, Bf^{F1}=0.01774

X^2=0.3385 $.95 < p < .98$ for 3 df

TABLE 4. Distribution of Bf phenitypesin some Caucasian populations (typed by HVAGE and immunofixation)

No. of cases	Population (Authors)	Bf^{S}	Bf^{F}	$Bf^{S0.7}$	Bf^{F1}	other
592	Veneto(this study)	0.73986	0.22804	0.01436	0.01774	-
1000	Tuscany(Domenici 1985)	0.7130	0.2495	0.0254	0.0130	-
400	Sicily(Crinò 1985)	0.8063	0.1688	0.0212	0.0025	0.0012
191	North-West.Italy (Malavasi 1981)	0.746	0.237	0.010	0.007	-
128	North-East.Italy (Malavasi 1981)	0.785	0.180	0.016	0.019	-
123	Central Italy (Malavasi 1981)	0.679	0.280	0.033	0.008	-
165	Southern Italy (Malavasi 1981)	0.715	0.258	0.018	0.009	-
217	Sardinia(Davrinche 1984)	0.5783	0.2189	0.0046	0.1982	-
1660	Sweden(Hjalmarsson 1981)	0.8139	0.1735	0.0042	0.0084	-
300	Norway(Teisberg 1977)	0.817	0.172	0.007	0.005	-
1245	Western-Germany(Mauff 1975)	0.8084	0.1743	0.0092	0.0077	0.0004
522	Germany-Hessen(Kühnl 1978)	0.7998	0.1772	0.0163	0.0077	-
1005	Minnesota Whites (Dykes 1980)	0.7985	0.1870	0.0040	0.0105	-

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J Clin Invest 47: 2181-2191

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TABLE 1. C3 distribution in Veneto

PHENOTYPES	OBSERVED		EXPECTED	
	n	%	n	%
S	482	0.65135	482.43798	0.65194
FS	230	0.31081	229.30790	0.30988
F	27	0.03649	27.24817	0.03682
S-VF, F-VF, VF	1	0.00135	1.00595	0.00136
	740	1.00000	740	1.00000

$C3^S$=0.80743 , $C3^F$= 0.19189, $C3^V$=0.00068

X^2=0.0048 $.95 < p < .98$ for 1df

TABLE 2. Comparison of C3 allele frequencies in several Populations

No. of cases	Populations (Authors)	$C3^S$	$C3^F$	$C3^V$
740	Veneto(this study)	0.80743	0.19189	0.00068
650	Tuscany(Domenici 1985)	0.803	0.195	0.001
325	Rome(Scacchi 1979)	0.791	0.203	0.006
950	Sicily(Bonavita 1985)	0.815	0.184	-
1034	Finland(Arvilommi 1973)	0.829	0.170	-
2454	Norway(Teisberg 1970)	0.786	0.208	0.005
213	Sweden(Bronnestam 1971)	0.770	0.230	-
406	Demark(Dissing 1971)	0.816	0.182	0.001
2340	Germany(Rittner 1974)	0.787	0.202	0.010
1988	Germany(Argawall 1972)	0.784	0.215	0.004
1326	Germany(Geserik 1980)	0.813	0.183	0.003
889	Switzerland(Pflugshupt 1973)	0.773	0.221	0.006
818	Belgium(Hoste 1977)	0.811	0.186	0.003
961	Spain(Argawall 1972)	0.782	0.211	0.006
388	China(Zhao 1983)	0.996	0.003	-
29	American Negroes (Alper 1978)	0.90	0.10	-

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the run, each plate was fixed in 5% TCA, dried, and finally, stained with CBB-R250.

Determination of Bf phenotypes was carried out by high voltage agarose gel electrophoresis followed by immunofixation according to Dykes(12,13). Electrophoresis and gel buffer was a barbital buffer pH 8.6. Undiluted serum samples were electrophoresed at 20 V/cm for 3 hours; an efficent cooling system was needed.

For immunofixation a cellulose acetate strip, saturated with Anti-human Properdin Factor B serum was placed for at least 60 min on the gel.

The staining was performed with Coomassie Blue R250.

RESULTS AND DISCUSSION

Table 1 illustrates the observed and expected distribution of phenotypes and gene frequencies of 740 blood donors of Veneto population tested for C3. The population is in Hardy-Weinberg equilibrium with a chi-square=0.048 $0.95 < p < 0.98$ for 1 d.f.

In table 2 our gene frequencies are compared to those found in other populations with similar studies: there is good accordance with the results obtained by other Authors in European countries . The most common gene in the populations studied up to now is $C3^{S}$.

In Table 3 the distribution of Bf phenotypes and of Bf alleles in a sample of 592 unrelated individuals from Veneto region is shown. A good fit to the Hardy-Weinberg law was observed.

In Table 4 our gene frequencies are compared with other found in some Caucasian populations using the same technique of electrophoresis followed by immunofixation.

The most common gene in the population studied at present is Bf^{S} caracterized by a frequency ranging from 0.5783 (Sardinia) to 0.8084(Germany). $Bf^{S0.7}$ and Bf^{F1} show similar frequencies, always lower than Bf^{F};

The C3 and Bf systems are informative, the methods for their determination are simply-reproducible and of relatively low cost, so that they appear useful for both population studies and cases of disputed paternity.

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THE POLYMORPHISM OF Bf AND C3 IN THE POPULATION OF VENETO (ITALY)

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INTRODUCTION

A genetic polymorphism of human C3 was demonstrated by Wieme and Demeulene are in 1967 (25) and later, confirmed simultaneously by Alper and Propp, Azen and Smithies (2, 5). They used electrophoretic techniques either in agarose gel or starch gel. They described a system with two codominant alleles $C3^S$ and $C3^F$. Further studies revealed 22 less common variants which have been designated according to their electrophoretic mobility.
Bf also shows a polymorphism discovered by Alper et al. in 1972 (1) and then a number of rare alleles have been described.
The structural gene locus Bf is characterized by some alleles of which are known two common F and S, two less common F1 and S0.7 and some others less frequent in according to their electrophoretic relative mobilities(13).
Both these complement components also been examined from two points of view: the population genetics and the medico-legal application.
The pourpose of this paper is to present the results of a study of the distribution of C3 and Bf phenotypes in the Veneto population.

MATERIAL AND METHODS

Serum samples, 740 for C3 and 592 for Bf ,were obtained from healthy blood donors from Padua Hospital Blood Bank (Veneto Italy).
Samples were stored at -30°C prior to analysis and typed within 2-3 days.
High voltage gel electrophoresis , as pubblished by Tiesberg (23)and modified by Domenici et al. (11) was used for phenotyping the C3 component. It consisted of a continuous buffer system with barbital/ cal= cium lactate buffer.
Electrophoresis was carried out on glass plate covered with 1% agarose gel. Serum samples diluted in saline, were applied cathodally. At the end of

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Advances in Forensic Haemogenetics 2
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GENETIC HETEROGENEITY IN ITALY

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The studies carried out by several Authors on the distribution of genetic markers in Italy have shown a more or less genetic heterogeneity of the Italian population. At present, however, a detailed regional analysis of it is still standing out.

Therefore we started a research program on the genetic structure of the population of Italy taking into account the ethno-linguistic minorities as well.

In this note we present a review on genetic variability of the several components of the Italian population based on the analysis of some red cell and serum genetic markers, as well the demographical and historical informations.

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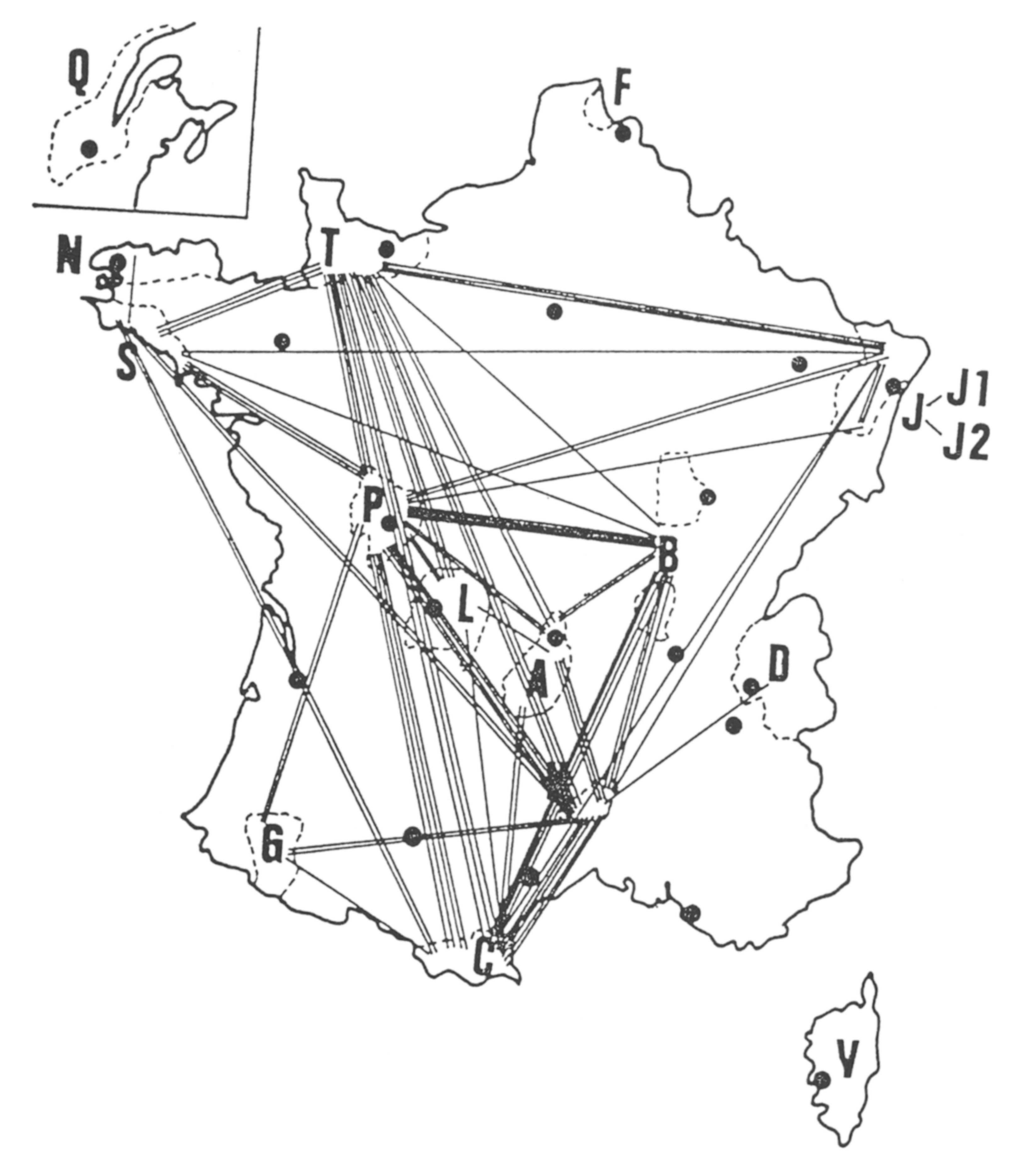

Fig 4. Genetic resemblances between French provinces.
The X^2 genetic distance was calculated for the following genetic systems : ABO, Rh, P, Kell, HLA-B, Gc, Pi, Tf
The number of lines between provinces is function of their genetic distances (1 : 0.07 ≤ d ≤ 0.08 ; 2 : 0.06 ≤ d ≤ 0.07 ; 3 : 0.05 ≤ d ≤ 0.06 ; 4 : 0.04 ≤ d ≤ 0.05 ; 5 : 0.03 ≤ d ≤ 0.04). The closest regions are C (Catalogne) and H (Cévennes). For province code letter, see text.

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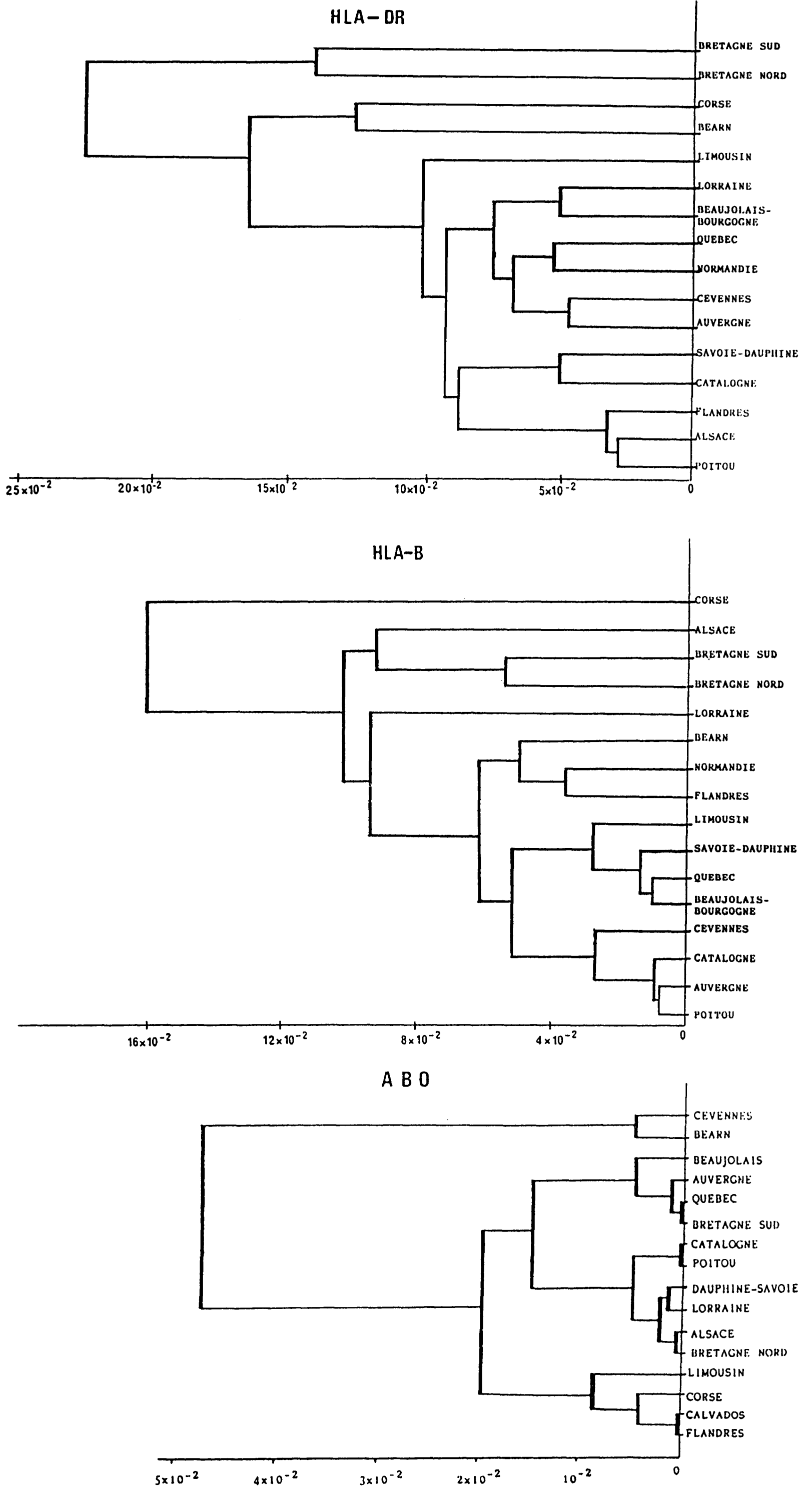

Fig 3. Dendrograms from X^2 genetic distances

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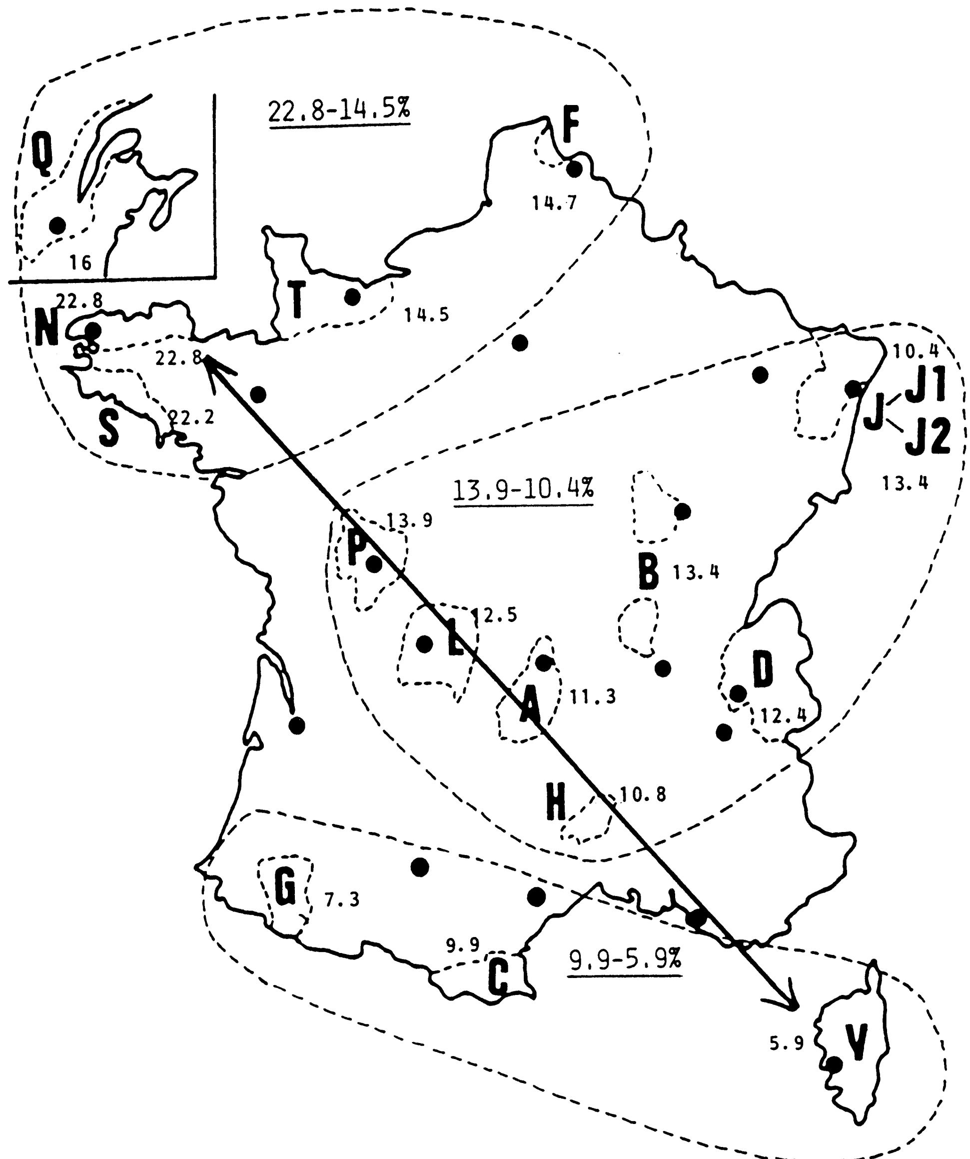

Fig 2. Distribution of HLA-DR4. Results are given as allele frequencies (%). For province code letter, see text.

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Table 4. Most frequent HLA haplotypes in the total sample "PF"

A1	Cw7	B8	C4AQ0	B1	BfS	DR3
A3	Cw7	B7	C4A3	B1	BfS	DR2
A29	Cw–	B44	C4A3	B1	BfF	DR7
A23	Cw4	B44	C4A3	B1	BfF	DR7
A2	Cw5	B44	C4A3	BQ0	BfS	DR4
A30	Cw5	B18	C4A3	BQ0	BfF1	DR3
A1	Cw6	B17	C4A6	B1	BfS	DR7
A3	Cw4	B35	C4A2,3	BQ0	BfF	DR1
A2	Cw3	Bw62	C4A4	B2	BfS	DR4
A2	Cw1	B51	C4A3	B1	BfS	DR2

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HLA-DR	N=2672	n=5437	n=5361
DR1	10.16	10.29	10.28
DR2	13.66	13.35	13.61
DR3	16.17	15.99	16.16
DR4	13.60	13.41	13.87
DR5	14.48	14.20	13.42
DRw6	10.06	9.91	8.75
DR7	15.17	14.99	15.26
DRw8	1.40	1.38	2.53
DRw9	1.00	0.98	0.73
DRw10	0.43	0.42	0.39
DR blank	3.87	5.07	4.98

BF	n = 5355
S	72.84
F	22.84
F1	3.07
S0.7	1.16
Rare	0.09

C4A	n=5188
A1	0.25
A2	5.18
A3	70.47
A4	5.57
A5	0.17
A6	3.43
AQ0	13.39
Rare	0.60
Duplication	0.94

C4B	n=5188
B1	71.32
B2	7.88
B3	1.06
B4	0.33
B5	0.21
B6	0.89
B7	0.10
BQ0	16.39
Rare	0.79
Duplication	1.04

GLO	n=4787
GLO 1	46.84
GLO 2	53.13
rare	0.02

N = number of unrelated individuals
n = number of haplotypes used in the family analysis
I allele frequencies (f) calculated from antigen frequencies in unrelated individuals (F) as $f = 1-\sqrt{1-F}$ and based upon local antigen assignment
II allele frequencies estimated by FAP from the same data
III allele frequencies estimated by FAP using the corrected data set after computer serological analysis.
BF, C4A, C4B and GLO allele frequencies were also estimated by FAP

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Table 3 - HLA allele frequencies (%) estimated by different methods and BF, C4A, C4B, GLO allele frequencies in the total sample

	I	II	III
HLA-A	N=2733	n=5526	n=5451
A1	13.77	13.37	13.64
A2	28.17	27.32	28.31
A3	13.18	13.14	13.28
A9	12.03	12.11	12.27
A10	4.34	4.24	4.32
A11	5.78	5.75	5.80
A29	6.61	6.75	6.58
Aw19.2	7.16	6.89	6.73
A32	3.27	3.27	3.35
Aw33	1.09	1.08	1.22
A28	3.67	3.68	3.67
A blank	0.93	2.40	0.83
HLA-C	N=2719	n=5527	n=5439
Cw1	3.55	3.73	3.70
Cw2	5.08	5.23	4.93
Cw3	8.95	9.24	9.89
Cw4	12.29	12.95	12.64
Cw5	8.90	9.25	8.74
Cw6	7.52	7.54	8.52
Cw7	20.10	20.31	22.39
C blank	33.61	31.75	29.20
HLA-B	N=2733	n=5505	n=5451
B5	8.00	8.17	8.21
B7	10.45	10.20	10.44
B8	10.04	9.81	9.99
B12	17.29	17.16	17.59
B13	1.57	1.58	1.61
B14	3.90	3.87	3.88
B15	6.23	6.15	6.16
Bw16	3.90	3.92	3.90
B17	4.12	4.07	4.10
B18	5.90	6.06	5.93
B49	2.17	2.21	2.07
Bw50	1.23	1.17	1.23
Bw22	2.40	2.36	2.40
B27	3.56	3.58	3.59
B35	9.21	9.16	9.61
B37	1.28	1.27	1.28
B40	5.50	5.42	5.06
Bw41	0.50	0.49	0.64
Bw47	0.38	0.40	0.42
Bw53	0.87	0.86	0.75
B blank	1.55	2.09	1.22

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Km N = 2720	Km(1) Km(3)	9.1 90.9
Gm N = 2718	1	68.2
	2	19.8
	3	8.7
	4	0.9
	5	0.8
	6	0.9
	7	0.1
	8	0.1
	9	0.2
	10	0.1
	11	0.1
	14	0.1

N = number of unrelated individuals
Var = rare variant
See text for definition of the genetic systems

Code	Gm haplotype nomenclature	
1	Gm^{4} ; ±23 ; 5*	Gm^{f} ; ±n ; b*
2	$Gm^{1,17}$; ; 21,28	Gm^{za} ; ; g^1g^5
3	$Gm^{1,2,17}$; ; 21,28	Gm^{zax} ; ; g^1g^5
4	Gm ; ±23 ; 5*	Gm ; ±n ; b*
5	Gm^{4} ; ±23 ;	Gm^{f} ; ±n ;
6	$Gm^{1,17}$; ; 5*	Gm^{za} ; ; b*
7	$Gm^{1,17}$; ; 5*,6	Gm^{za} ; ; b^*c^3
8	$Gm^{1,17}$; ; 10,11,13,15,16	Gm^{za} ; ; $b5b^0b3st$
9	Gm^{17} ; ; 21,28	Gm^{z} ; ; g^1g^5
10	$Gm^{1,2,17}$; ; 5*	Gm^{zax} ; ; b*
11	Gm^{4} ; ±23 ; 5*28	Gm^{f} ; ±n ; b^*g^5
12	$Gm^{1,17}$; ; 10,11,13,15,16,6	Gm^{za} ; ; $b5b^0b3stc^3$
13	Gm^{4} ; ±23 ; 21,28	Gm^{f} ; ±n ; g^1g^5
14	$Gm^{1,17}$; ; 5*28	Gm^{za} ; ; b^*g^5

5* = 5,10,11,13,14 b* = b^1,b^5,b^0,b^3,b^4

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Table 2. Allele or haplotype frequencies (%) for non MHC systems in the total sample "PF"

Genetic system	Allele or haplotype	Frequency %
ABO N = 2672	A	26.2
	B	6.0
	O	67.7
Rh N = 2665	DCe	42.7
	DcE	13.1
	Dce	2.0
	DCE	0.4
	dce	40.2
	dCe	0.9
	dcE	0.6
	dCE	0.0
MNSs N = 2633	M	52.2
	N	47.8
	S	32.8
	s	67.1
	MS	23.8
	Ms	28.4
	NS	8.4
	Ns	39.4
P N = 2650	P1	53.1
	P2	46.9
Kell N = 2667	K	4.2
	k	95.8
Pi (α_1-anti trypsin) N = 2707	M1	61.3
	M2	15.3
	M3	9.9
	M4	1.4
	M5	0.6
	S	9.6
	Z	1.4
	Var	0.5
Gc (DBP) N = 2713	1F	15.6
	1S	53.9
	2	29.9
	Var	0.6
Hp N = 2687	Hp1	38.4
	Hp2	61.6
Tf N = 2706	C1	75.7
	C2	19.0
	C3	4.5
	C Var	0.1
	D	0.1
	B	0.6

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Table 1. Quality of HLA-antigen definition

Excellent	Good	Rather Good	Poor
A1	A28	A30	A31
A2	A32	Aw33	Aw66
A3		Aw34	
A29	Cw1		Bw41
A11	Cw2	Cw6	Bw58
A23	Cw3	Cw7	Bw61
A24	Cw4		
A25	Cw5	B38	DRw8
A26		B45	DRw9
	B18	Bw47	DRw10
B7	B35	Bw52	DRw12
B8	B37	Bw53	DRw14
B13	B39	Bw60	DQw2
B14	Bw50		
B27	B51	DR1	
B44	Bw55	DR3	
B49	Bw56	DRw13	
Bw62	Bw57	DQw1	
Bw63	Bw4	DQw3	
	Bw6		
DR7			
	DR2		
	DR4		
	DRw11		
	DRw52		
	DRw53		

The quality of antigen definition was assessed according to the correlation coefficient (r) between computer and laboratory antigen assignment, the quality score (Q score) the normalized Q score (N-Q score) and the number of sera used (Sierp et al 1986).

Excellent : $r \geq 0.96$ and at least 2 excellent sera
Good : $r \geq 0.91$ and at least 3 good or rather good sera
Rather good : $r \geq 0.75$ and at least 2 good or rather good sera
Poor : $r < 0.75$ and/or less than 2 rather good sera

Excellent sera are defined by a N-Q score ≥ 0.5 and Q score value ≥ 8,
Good sera are defined by a N-Q score > 0.5 and Q score value 4-8,
Rather good sera are defined by a N-Q score 0.25 to 0.49 and Q score value ≥ 4.

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COMMENTS AND CONCLUSION

The FAP program gave the most accurate estimation of gene and haplotype frequencies. However as shown in Table 3 the advantage of using a family analysis program is not so much for allele frequencies estimations, which vary only slightly according to the methods used ; it is mainly for haplotype and linkage analyses. This family material allowed to consider extended HLA haplotypes and complotypes ; the analysis of recombinations gave recombination fraction values (ϑ) concordant with those of the litterature : HLA-A/C : 0.47 and 0.95, HLA-C/B : 0.06 and 0.09, HLA-B/BF : 0.47 and 0.61, HLA-DR/BF : 1.13 and 0.94, HLA-DR/GLO : 5.72 and 11.16 for paternal and maternal ϑ values respectively.

When performing inter-regional comparisons, a remarkable genetic heterogeneity was found and allowed to define regional genetic characteristics. The sample from Corsica was very different from all the others ; although the general picture of gene frequency gradients for individual genetic systems is quite complex a clear difference between the provinces of Northern France and those of Southern France is constantly found.

Numerous other analyses will be done in the future as this study has produced one of the largest available data set on genetic markers based on a family material homogeneously studied. But one must be careful not to overinterpret the results in terms of history or population migrations.

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possible genotypes or haplotype combinations compatible with the given phenotypes in each family ; it detects possible recombinations. It is therefore extremely helpful for analyzing multiple loci systems : it allowed to estimate the frequency of 8 locus haplotypes : HLA-A,C,B,BF,C4A,C4B,DR,GLO, as well as allele frequencies, to estimate the recombination fraction and to calculate 2 locus linkage disequilibrium.

- Hardy-Weinberg equilibrium was tested for all systems.

3. Inter-Regional Analysis

As the gene frequencies showed strong differences between provinces a number of inter-regional analyses were undertaken. We will only show here results of genetic distances calculated according to Balakrishnan and Sanghvi (1968). Other indexes of genetic distances have been used (Cambon-Thomsen et al 1986) and a principal component analysis leading to generate coloured synthetic maps was performed by A. Piazza (1986).

SOME RESULTS OF THE PF ANALYSIS :

Table 1 shows the quality of HLA antigen definition as result of the serological analysis.

Table 2 gives the gene frequencies of non MHC markers estimated by maximum likelihood from the phenotypes of the parents, on the total sample.

Table 3 compares the HLA allele frequencies obtained by different methods from the total data set : (I) calculated from the antigen frequencies and based upon local antigen assignment, (II) estimated by FAP from the same data, (III) estimated by FAP using the corrected data set after serological analysis by computer ; allele frequencies for BF, C4A, C4B and GLO were also estimated by FAP.

Table 4 shows the most frequent HLA haplotypes in the overall sample by order of decreasing frequencies.

Figure 2 gives an example of an HLA-allele distribution (HLA-DR4) : there are strong differences between provinces with frequencies (%) decreasing according to a North-West/ South axis.

Figure 3 shows three dendrograms drawn from the matrix of X^2 genetic distances for three loci (ABO, HLA-B and HLA-DR).

Figure 4 illustrates the genetic resemblances between French provinces derived from the matrix of genetic distances calculated for a group of independent genetic systems (ABO, Rh, P, Kell, HLA-B, Gc, Pi, Tf). The number of lines between provinces is maximum when the distance is minimum.

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The control of data and data entry was done at two levels : **1)** by the regional coordinator in each province, upon receipt of a clear listing of the data entered ; **2)** by the centralizing team using both computer programs and human ability to correct the considerable amount of logical and clerical errors which are unavoidable when data files consist of contributions from numerous laboratories.

DATA ANALYSIS

This analysis was performed in several steps before the data bank was made available for the scientific community : the final data set and the estimation of the population genetics parameters are the fruit of an international collaboration between INSERM U 100, Toulouse ; CRPG-CNRS, Toulouse ; Institute of Medical Statistics, Bonn and Immunogenetics Laboratory, Munich.

1. HLA Serological Analysis

The programs were derived from those used in 1984 for the 9th international histocompatibility workshop (Albert et al 1984, Deppe et al 1984). Based on the serological reaction scores from the 192 common alloantisera and on the laboratory HLA antigen assignment they allowed to evaluate the quality of the serums (Q scores), the quality of antigen definition (Table I) and to perform a computer cell typing. The overall correlation coefficient between laboratory and computer antigen assignment was equal to 0.93. After this analysis a new set of HLA phenotypes was created for all individuals tested for further genetic analyses.

2. Population Genetics Analysis :

Two approaches were used for the estimation of gene frequencies in each province and for the total sample :

- without considering the family information, the antigen and phenotype frequencies were calculated from the unrelated individuals (parents) by a simple counting method and the gene frequencies were estimated by maximum likelihood ; for HLA genes the formula $f = 1-\sqrt{1-F}$ where f is the gene frequency and F the antigen frequency was used.

- with family analysis, using the set of programs FAP originally written for the 9th international histocompatibility workshop in 1984 and adapted for this analysis (Neugebauer et al 1984, Borot et al 1986). This program was first used for analyzing the MHC markers on **a)** data from laboratory HLA antigen assignment, **b)** corrected data after computer serological analysis ; then it was adapted for application to all the other systems and this analysis is currently being completed. This program is excellent for checking family data consistency in large data sets ; it estimates the likelihood of all

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Erythrocyte blood groups :

Typing for ABO (ch 9), Rhesus (ch 1), MNSs (ch 4), P (ch 22) and Kell was done by classical haemagglutination methods in the laboratories of each province.

Major histocompatibility complex (MHC) : the human MHC is the most polymorphic genetic system with polyallelic multiple loci and is very informative in population genetics (Baur and Danilovs 1980 ; Greenacre and Degos 1977 ; Ryder et al 1978). It is located on the chromosome 6 and includes HLA genes (HLA-A,B,C,DR,DQ,DP) and non HLA genes (some complement genes, 21-hydroxylase genes, etc...). An erythrocyte enzyme, the glyoxalase-I (GLO) is also coded nearby this region. The HLA antigens HLA-A,B,C and DR were determined by lymphocytotoxicity in the regional laboratories with the help of a common set of 192 alloantisera previously selected by 8 of the laboratories. The complement allotypes BF, C4A and C4B were studied by electrophoresis and immunofixation mainly in two laboratories (G. Hauptmann, Strasbourg and M. Abbal, Toulouse) although 5 other laboratories also performed this typing for some provinces. Finally GLO was studied by electrophoresis principally by J. Arnaud (Toulouse) and M. North (Strasbourg), and samples from 3 provinces were studied in other laboratories.

Serum proteins

All the following systems were studied centrally for all provinces in one laboratory in Toulouse, Centre de Recherches sur le Polymorphisme Génétique des Populations Humaines (CRPG), CNRS :

Immunoglobulin allotypes of systems Gm (ch 14) and Km (ch 2) were determined by inhibition of haemagglutination (M. Blanc).

Serum proteins with electrophoretic polymorphism were investigated by J. Constans : Protein inhibitor (Pi) or $alpha_1$ antitrypsin (ch 14), Group Specific Component (Gc) or vitamin D binding protein (DBP) (ch 4), haptoglobin (Hp) (ch 16) and transferrin (Tf) (ch 3). Pseudocholinesterase polymorphism (ch 1) was tested by J. Arnaud.

A detailed description of these genetic systems, the distribution of their alleles in populations and the detailed results in PF may be found in Ohayon and Cambon-Thomsen (1986, 1987).

6. Data Entry and Control of Data Set :

The data entry was centralized by A. Sevin (CNRS, Toulouse). The common coding forms allowed to create two kinds of data files : **1)** a data set per family, based on the answers to a questionnaire giving origin, location, identification (number) and pedigree of the family along with some socio- cultural and medical information ; **2)** a data set of laboratory results per individual with HLA serological reaction scores and local

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3. Choice of Families :

The rule was to choose families who had been settled in the province for at least three generations. For this reason most of the families lived in rural areas where the migration rate is low. Each family should be available for answering a questionnaire and for giving a blood sample from at least the 2 parents and 2 children over 10 years. Within each province the families were contacted with the help of blood banks, schools, articles in local journals, local administrators, social organizations and also through the families previously contacted especially for completing the sample at the end of the survey.

4. Sample Size :

The number of 100 unrelated families per province was chosen after calculating the probability not to observe an allele of frequency 1% according to the sample size. This probability is between 1 and 2% for a sample of 200 individuals. Figure 1 presents the distribution of the 1382 families among the provinces studied. The analysis could be done for 1360 of them, which represents a total of 5500 persons with 2720 unrelated individuals. An important fact is that the families were selected according to given criteria and, consequently, were not random. The total sample (all provinces together) is not intended to represent "the" French population and this study was mainly done for the comparison of samples of approximatively the same size. Fourty-three to 106 families were studied per province. The low number of families in Limousin (43), Savoy-Dauphiné (54) and Corsica (76) implies that the results in terms of frequencies should be considered with caution. At the beginning of the study Alsace and Lorraine were put together with around 50 families in each. But at the time of the analysis we considered them independently because a strong genetic heterogeneity appeared. The North-Western part of France is slightly overrepresented : 414 families, as compared to 232 from the South-Western part, 311 from the North-East and 335 from the South-East). This is also explained by the fact that some regions like Aquitaine (South-West) adn Provence (South-East) which would have been interesting to study were not considered because none of the laboratories could perform the survey and the typing for these provinces. Thus our sample may be representative of a stable rural population of some French provinces at the beginning of this century ; but one of the major interests is to provide us with genetic data on a large sample of families.

5. Panel of Genetic Markers :

The following genetic systems were studied and we indicate between brackets the chromosome (ch) where the corresponding loci are located :

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geographical and historical criteria. Fifteen French provinces were chosen and a sample of Canadians of French origin from Quebec was also included in the study. These provinces designated by a code letter were studied by the different volunteer laboratories under the responsibility of a regional coordinator (name between brackets) : F, Flanders (F. Dufossé), J1, Alsace (M.M. Tongio), J2, Lorraine (P. Perrier), B, Beaujolais-Burgundy (H. Bétuel, F. Guignier), D, Savoy-Dauphiné (J.C. Bensa), V, Corsica (J.P. Amoros, P. Mercier), H, Cévennes (J. Constans, J.Y. Muller, J. Seignalet), C, Catalonia (J. Hors, Ph. Lefèvre-Witier), G, Béarn (M. Calot), L, Limousin (G. Malinvaud), A, Auvergne (C. Moennard), P, Poitou (D. Alcalay), S, South Brittany (R. Fauchet), N, North Brittany (J.P. Saleun), T, Low Normandy (A.M. Griveau), Q, Quebec (F. Décary). Each regional coordinator was responsible for defining the precise geographical limits of the province, which could be divided in "sub- regions", and for finding the families homogeneously distributed over the whole province.

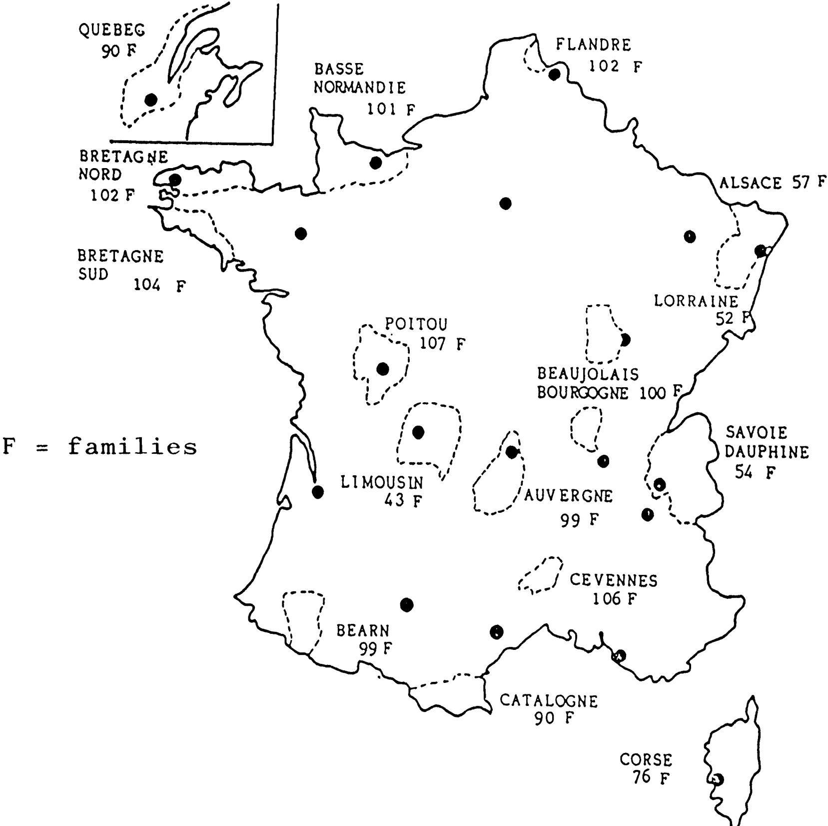

Fig 1. Distribution of the families studied in the genetic survey "Provinces Françaises". The names of the provinces are given in French. The total number of families is 1382; 1360 of them could be fully analyzed.

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BASES OF THE COLLABORATIVE STUDY "PROVINCES FRANCAISES"

Since Landsteiner discovered the first blood group system (ABO) in 1900, the study of genetic markers has become a large part of human population genetics. Variable gene frequencies were found in different populations and genetic distances could be calculated and compared to geographical data (Cavalli-Sforza and Bodmer 1971 ; Mourant 1985 ; Piazza and Menozzi 1983 ; Salmon et al 1984). However new interests in such studies arose about 15 years ago as 1) new genetic systems with higher polymorphism were discovered, 2) some of them were shown to have a fundamental biological role, such as the HLA system regarding immune response and 3) genetic epidemiology began to be recognized as important and brought population genetics to the field of medicine (Degos et al 1977 ; Morton 1982).

It is well known that the French population has a heterogeneous origin and a complicated history and previous studies have already demonstrated that genetic markers are not evenly distributed between regions (Mayer et al 1981 ; de Mouzon et al 1980 ; Prévost et al 1984 ; Vergnes et al 1980 ; Youinou et al 1983). However there were no homogeneously established data in France on the distribution of genetic markers in different regions, based on family studies. We therefore undertook this work in order 1) to describe the distribution of gene frequencies in the French provinces, 2) to organize a data bank which could be useful in different fields (human genetics, anthropology, history, demography, medicine and epidemiology), 3) to produce reference maps of gene frequencies 4) to allow the study of inter-regional variability in France on the basis of genetic data.

METHODOLOGY OF THE SURVEY "PROVINCES FRANCAISES"

The originality of this study mainly consists in the methodology : it is a prospective study, with homogeneous methods (within the limits imposed by a multicenter study) both for the choice of the samples and for the reagents and techniques used. The principal steps were the following :

1. Call for Participants :

This was done in 1980 and 20 laboratories were willing to participate. They became a determining element in defining the size, the location and the number of population samples which could be studied.

2. Choice of Areas :

The rules were to cover as large a part of France as possible, to avoid migration axes and zones of known population admixture as well as isolates and to take into account

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Practical Application of Population Genetics : the Genetic Survey "Provinces Françaises"

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INTRODUCTION

From 1981 to 1985, a genetic survey in the French provinces and in Quebec was conducted by more than 20 laboratories specialized in immunogenetics and human genetic polymorphism studies, under the coordination of E. Ohayon and A. Cambon-Thomsen. A large panel of genetic markers was studied in 1382 families of precisely known French origin ; the data bank was completed and population genetics analyses were performed between 1985 and 1987.

In this report we will describe the bases of this study, the methodology of the survey with comments about the samples of population studied, the organization of the data bank "PF" (for "Provinces Françaises") and the methods used for the population genetics analysis. Some of the results will be presented but more details about this collaborative work may be found in several volumes published by INSERM, Paris (Ohayon and Cambon-Thomsen 1986, 1987).

**
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POPULATION GENETICS: MATHEMATICAL PROBLEMS

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The use of polymorphic marker systems in paternity testing and the quantification of the contained information by way of a posterior probability or likelihood ratio is based on the knowledge of the formal genetic model for the given polymorphism, i.e. the genotype(s) - phenotyp relationship, and the population genetic parameters contained in the gametic as well as genotypic frequencies. The likelihood calculation, which generally uses simplifying assumptions regarding such evolutionary forces as selection, migration and deviation from Hardy-Weinberg equilibrium, depends on the availability of gametic frequencies from population studies. Gene frequencies from single locus marker systems with known mode of inheritance are straight forward to estimate by way of genecounting or maximum-likelihood estimation. The introduction of closely linked loci into paternity testing creates numerous problems by way of additional parameters such as linkage disequilibria and recombination fractions. Whereas the power of such highly polymorphic systems is desirable, its quantification can only be achieved by way of large samples of unrelated individuals and in complicated situations only by way of population studies on the level genotyped parents from random families.

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This very short introductory remarks should show the extreme complexity of population genetics. Many problems which arise during the study of populations are due to the facts that the true populations are finite in size, that generations overlap and that the evolutionary forces vary rapidly. It is clear that this complexity has some influence of the gene frequencies which are currently used for the biostatistical evaluation of paternity; in the practical use, however, if enough genetic markers - especially the HLA system - are used, these factors will not influence the biostatistical opinions in a major degree.

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selection against heterozygotes, existence of a silent allele (which makes homozygotes indistinguishable from heterozygotes carrying this silent allele), misclassification of phenotypes or positive assorting mating (tendency of individuals with similar phenotype to mate with each other); an excess of heterozygotes can be due to: selection in favour of heterozygotes, errors in the classification of phenotypes or negative assorting mating.

The Hardy-Weinberg law can be easily extended to multiple alleles, polyploids, sex-linked genes or several closely linked loci. Evolution can be seen through the changes of the frequencies of the genes. The major forces of evolutionary processes are: natural selection, mutation, migration and random genetic drift.

Random genetic drift is the random change of gene frequencies in a population with small size. Without selection, mutation or migration, the ultimate outcome of random genetic drift is the fixation of one allele and the disappearence of the other ones. In humans, migrations can be subdivided in at least three kinds: individual migrations, massive migrations and demic expansions (migration and extreme population growth). Migration is the major force against the effects of random genetic drift. The main factors with regard to evolution are mutation and selection. Mutation produces new alleles and, therefore, is the basis for the development and existence of polymorphisms (i.e. the state in which a mutant gene is rather frequent - frequency higher than 0.01 - and in which more than one phenotype can be distinguished). Most of the mutations, however, are deleterious, so that they are selected against. At equilibrium, a balance between mutation and selection (equilibrium between new mutations and elimination of the mutants by selection) exists. This equilibrium depends on several factors, e.g. whether the mutant is autosomal or sex-linked, or on the fitness of the genotypes carrying the mutant genes. In some cases, heterozygotes for a normal and a mutant gene show in a given environment a higher fitness than homozygote carriers of the normal allele. The best known of such situations is the relationship between the sickle-cell polymorphism and malaria which is an excellent example for a balanced polymorphism with heterozygote advantage.

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Population genetics, a short introduction

W.R. Mayr

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Population genetics is mainly concerned with the study of the nature and the source of inherited differences of individuals belonging to the same species (for review, see e.g. Cavalli-Sforza and Bodmer 1971). Investigations in population genetics analyse the changes of the frequencies of the different phenotypes found in a population and the conditions causing an equilibrium between the various forces influencing these phenotypes.

Populations can be described by the distribution of the frequencies of the various phenotypes, that means by the gene frequencies in the different polymorphisms.

In Mendelian populations, i.e. infinitely large populations of interbreeding subjects with a common pool of genes, these genes are transmitted from one generation to the other according to the laws discovered by Gregor Mendel in 1865. The genetic composition of such populations undisturbed by evolutionary forces will not change and correspond to the Hardy-Weinberg law. This Hardy-Weinberg law shows that under random mating a biallelic autosomal locus with the genes A_1 and A_2 (relative frequencies of the alleles: p_1 and p_2) reaches in one generation a distribution of the genotype frequencies given by the expansion of the formula $(p_1 + p_2)^2$, that means the terms p_1^2, $2\ p_1p_2$ and p_2^2 which give the relative frequencies of the genotypes A_1A_1, A_1A_2 and A_2A_2, respectively. Based on the phenotype frequencies, the allele frequencies can be calculated by using the Hardy-Weinberg law.

Departures from the Hardy-Weinberg equilibrium are rare, but can be due to several reasons. A deficiency of heterozygotes is sometimes seen in the following cases: heterogeneity of the population (e.g. very recent mixture of populations), inbreeding,

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VI. Population Genetics

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Alkohol- und acetonfixierte Gewebsproben erbrachten gleichmäßige, gut ablesbare Zymogramme im GLO- und PGM-System. Ein Waschen der Gewebsproben nach Fixierung, vor Probenentnahme, führte zu einer Abschwächung der Banden im PGM-System in der Stärkegel-elektrophorese, besonders im Bereich der 2er-Spots.
Nach längerer Lagerungszeit der Gewebsproben in Alkohol zeigte sich eine deutliche Abschwächung der Ergebnisse, so daß das Aceton für die vorliegenden Untersuchungen als ideales Fixationsmittel anzusehen ist.
GLO- und mit Einschränkungen GPT-Muster der Gewebe entsprechen dem Bild der Erythrozyten-Hämolysate, daß jedoch die PGM-Muster vom Gewebe etwas anders ausfallen als die der Erythrozyten-Hämolysate war nach den Erfahrungen von Oepen am unfixierten Muskel in der Stärke-Gel-Elektrophorese zu erwarten. Im wesentlichen zeigte sich in vorliegender Untersuchung ein abweichendes Bild im Bereich der b- bzw. c-Bande. Ähnlich wie von Berg e.a. an gelagerten Blutproben und Blutspuren beschrieben, zeigte sich ein anodales Auswandern der c-Bande.
Bei zunehmender Abschwächung der Banden konnten GLO und GPT bis zu einem Zeitraum von 4 Wochen typisiert werden, das PGM-System ergab nach einer Lagerungszeit von 2 Monaten noch gut ablesbare Ergebnisse.

ZUSAMMENFASSUNG

Zusammenfassend ist festzuhalten, daß bei traumatisierten, ausgebluteten Leichen mit der aceton- und aethanolfixierten Skelettmuskulatur Untersuchungsmaterial zur Verfügung steht, woran sich serologisch interessante Systeme auch nach längerer Lagerungszeit mit der im Strafprozeß erforderlichen Sicherheit nachweisen lassen.

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Da, wie angeführt, ein Enzymnachweis am frischen Gewebe oder Gefrierschnitt nur über kurze Zeit möglich ist, sollte, um zumindest eingeschränkt zeitunabhängig arbeiten zu können, eine Fixationslösung gefunden werden, wodurch die Enzymaktivität serologisch interessanter Enzymsysteme (EsD, GLO, GPT, SEP, PGM) nicht zerstört wird.

Zahlreiche Untersuchungen sind im Rahmen der Histochemie auf die Ermittlung der nach Fixation verbleibenden Aktivität verschiedener Enzyme gerichtet worden, wobei Ziel der Fixation sein sollte, die Enzyme unlöslich zu machen, ohne die Aktivität merklich zu beeinflussen. Auch muß das Enzym nach der Fixation außer der Aktivität noch seine Spezifität besitzen. Empfohlen wird zur Fixation Aceton p.a. sowie Aethanol, da diese Reagenzien vom chemischen Gesichtspunkt wenig aggressiv erscheinen. Formalin und Glutaraldehyd sollen bei einigen Enzymen, u.a. der sauren Phosphatase, dem Aceton und Aethanol überlegen sein.

MATERIAL UND METHODE

Anläßlich gerichtlicher Obduktionen wurden Gewebsblöcke, 2x 1 cm, aus m. psoas und Leber entnommen, wobei darauf geachtet wurde, daß die Leiche keine offensichtlichen Fäulniserscheinungen aufwies, ferner wurde eine Kontrollblutprobe asserviert.
Als Fixationslösungen wurden Formalin 4 %, mit NaOH gepuffertes Formalin pH 7,5, Glutaraldehyd 6,5 % (Sacharoselösung 0,2 M), Aethanol absolut und Aceton p.a. verwandt.
Von den fixierten Gewebsblöcken wurden mit dem Skalpell kleinste Teile (5 mg) abgeschnitten und auf einer Glasplatte mit einem Reagenzröhrchen zu dünnen Plättchen ausgerollt. Durch Verdunstung entstanden harte Plättchen, die auf die Größe der üblicherweise verwendeten Filterpapiere zugeschnitten wurden und danach direkt auf das Gel aufgelegt bzw. in das Gel verimpft wurden.
Folgende Enzymsysteme wurden in die Untersuchung mit einbezogen: EsD (Agarose-Gel-Elektrophorese), GLO, EsD, SEP (Stärke-Gel-Elektrophorese), PGM (Stärke-Gel-Elektrophorese und Isoelektrofokussierung).

ERGEBNISSE

Die in Formalin 4%, gepuffertem Formalin und Glutaraldehyd fixierten Gewebsproben erbrachten in keinem der angeführten Systeme ein Ergebnis, die Enzymaktivität war offensichtlich rasch zerstört worden.
Die saure Erythrozyten-Phosphatase und Esterase-D waren am Muskel und der Leber nach Fixation nicht mehr nachweisbar.
Die Ergebnisse im GPT-System am aceton- und alkoholfixierten Muskel waren bei gleichbleibenden Untersuchungsbedingungen stark schwankend, teils zeigten sich überfärbte, teils zu schwache Banden. Die Leberproben ergaben hier durchgehende Schlieren, keine abgrenzbaren Spots, so daß eine Typisierung nicht erfolgen konnte.

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Enzymdarstellung am fixierten Organgewebe

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SUMMARY

The successful determination of GLO subtypes (starch gel electrophoresis)and PGM subtypes (isoelectrofocusing) in tissues,skeletal musculature and liver, applying ethanol and acetone fixation is reported.
Other fixing agents such as formalin, buffered formalin pH 7,5 and glutaraldehyde are destroying any enzyme activity of the considered systems EsD, GLO, GPT, SEP, PGM.
Applying ethanol and acetone fixation no results could be obtained in EsD and SEP system.
Differing results showed GPT, the intensity of the spots varied extremely even under stable experimental conditions.
GLO and PGM subtyping using ethanol was successful within a period of four weeks. PGM subtypes using acetone fixation could be demonstrated even after a storage time of two months.

EINLEITUNG

Die Anforderungen der Strafverfolungsbehörde an die Aussagekraft eines serologischen Spurengutachtens sind hoch, wobei der Gutachter nicht selten vor dem Problem steht, daß aufgrund ausgedehnter Verletzungen oder fortgeschrittener Fäulnis bei der Obduktion von Getöteten kein Blut zur Blutgruppenbestimmung gewonnen werden kann, somit eine Zuordnung von Spuren nicht möglich erscheint. Über den Nachweis von Blutgruppensubstanzen an Sekreten und Körpergeweben existieren zahlreiche Arbeiten, wobei die Untersuchungen am Gewebe überwiegend entweder am frisch entnommenen Material oder an Gefrierschnitten durchgeführt wurden (Hoste, e.a.,Oepen, Oya, e.a., Stöhlmacher, e.a., Tutsch-Bauer, e.a.). In der umfangreichen Darstellung "Zur Blutgruppenprägung menschlicher Körpergewebe" wurde von Oepen vermerkt, daß über Muskulatur relativ wenig berichtet wurde, obwohl sie neben guter Resistenz gegenüber autolytischen Prozessen vor allem auch von Extremitäten gewonnen werden kann. Bei Leichenzerstückelung oder Massenkatastrophen kann somit durch Bestimmung von Blutgruppen- und Enzymsystemen am Muskel eine Zuordnung von Leichenteilen bzw. eine Identifizierung erfolgen. Von Oepen wurde weiter ausgeführt, daß die Untersuchung bereits fixierter Organe, die offenbar erfolgreich sein kann, in der Praxis kaum Anwendung findet.

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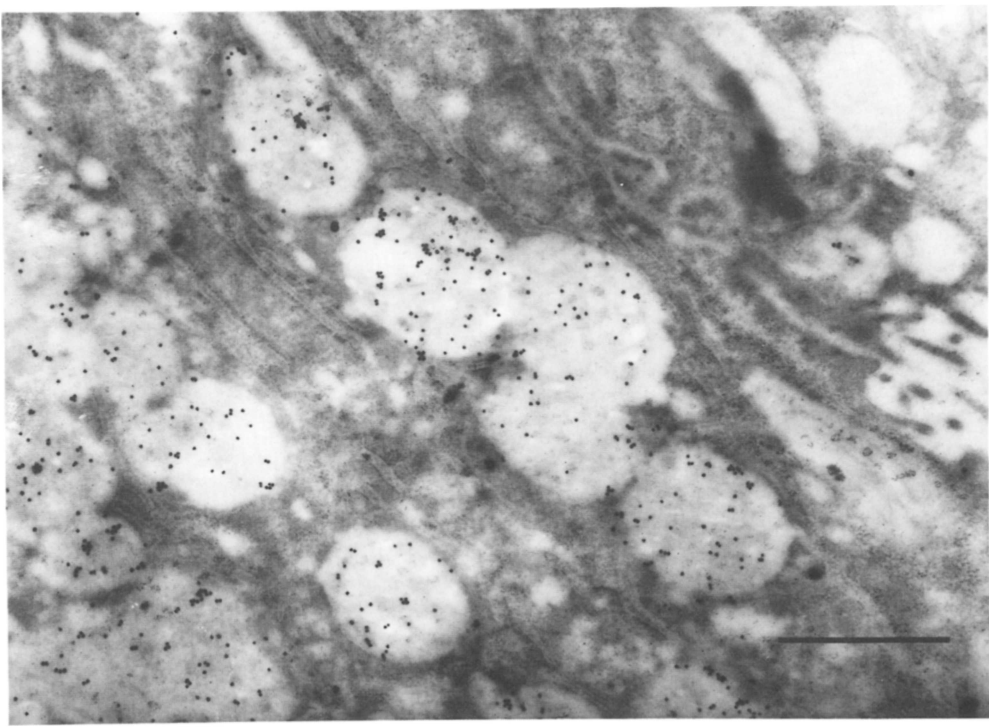

Fig. 2. Secretory cells from a blood-group A secretor incubated with DBA-gold complexes. Secretory granules are intensely labeled. Original magnification x16000, Bar= 1μm.

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Table 3. Lectin-HRP staining of cervical glands in human uteri

Blood group		Case	Lectin used in this study						
			DBA	GSI-B_4	UEA-I	SBA	PNA	RCA-I	GS-II
A	S	17	+1∽+2	-∽+2	+1∽+2	+1∽+2	+1∽+2	+1	-∽+1
	NS	2	-	-	-	-∽+2	-∽+2	+1∽+2	-
B	S	4	-	+1∽+2	+2	+1	+1∽+2	+1	-
	NS	1	-	-	-∽+1	-	+1	+2	-
O	S	11	-	-	+1∽+2	-∽+2	+1∽+2	+1∽+2	-∽+1
	NS	1	-	-	-∽+1	+1	+2	+1	-
AB	S	4	-∽+1	+1∽+2	-∽+2	+1	+2	+1∽+2	-∽+1
	NS	2	-	-	-∽+2	-∽+1	+1	+1	-

Numbers indicate the staining intensity. -, no staining; +1, weak; +2, moderate; +3, strong. S, secretor; NS, nonsecretor

reacted with secretory cells regardless of the blood group of the tissue donors. PNA reacted moderately with the cervical glands, while SBA tended to react more strongly with secretory cells of blood group A secretors than those of other blood groups. GS-II did not react with secretory cells of cervical glands.

Generally, these blood group spicific as well as nonspecific lectins reacted uniformly with the secretory cells of the cervical glands of any blood group. However, in luteal phase they tended to exhibit stronger reactivity in the apical pole of the secretory cells. These results suggest that the patterns of lectin staining are likewise dependent on the phase of menstrual cycle as found in conventional mucin staining.

3.Electron Microscopic Observation of Lectin-binding Sites

DBA, GSI-B_4, and UEA-I-gold complexes reacted intensely with the secretory granules(Fig.2). Other cytoplasmic organelles such as nuclei, mitochondria, endoplasmic reticula and the Golgi complexes were not labeled at all with these lectin-gold complexes. Reactivity with these lectin-gold complexes was also strictly dependent on the blood group and secretory status of the tissue donors.

In conclusion, labeled lectins are reliable histochemical reagents for demonstrating the precise distribution of blood group antigens and their related substances at both the light and electron microscopic level in human cervical glands.

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Fig 1. Schematic drawing to show the staining patterns of cervical glands with aging and during menstrual cycle

Stadium Juvenile	Stadium Senile	Stadium Maturum			
		Follicular	Ovulation	Luteal	Menstrual

also stained most of secretory cells, however, some cells often did not react with HID and HID-AB sequence resulted in blue tincture of these cells. Similar results were obtained with AF staining. Digestion with neuraminidase did not affect the staining intensity with AB pH2.5. These results demonstrate that cells in the cervical glands secrete mainly neutral and sulphated mucopolysaccharide. Sialic acid susceptible to neuraminidase digestion can not be detected in the present study.
Next, we examined the changes in staining patterns of the cervical glands with aging and during menstrual cycle(Fig.1). The secretory cells in the stadium juvenile and stadium senile were usually stained uniformly by the methods employed. During menstrual cycle the staining patterns changed, depending on the phase of menstrual cycle, namely secretory cells in the follicular phase and the ovulation phase were usually stained uniformly, while, in the luteal phase, many cells exhibited the reactivity with these stains only at the upper half of the cells, and in the menstrual phase, many cells exhibited the reactivity with these stains only in their apical pole.

2.Lectin-HRP Staining

The results are summarized in Table 3. The staining patterns of blood-group specific lectins showed strict dependence on the blood group of the tissue donors. DBA reacted with the cervical glands of blood group A and AB secretors but did not react with those of blood group B and O, and nonsecretors of any blood group. GSI-B_4 also reacted specifically with the glands of blood group B and AB secretors. UEA-I reacted with the cervical glands of any blood group of secretors and weakly reacted with those of blood group B,O,AB nonsecretors but not with those of blood group A nonsecretors. The distribution of cells reactive with these lectins was not homogeneous in blood group A,B,AB donors. For example, in some glands of blood group A donors, only a limited number of cells were stained with DBA, while almost all the cells were stained with UEA-I. Cells, which reacted with DBA but not with UEA-I, were also observed. In blood group B and AB donors, similar mosaic distribution of lectin-reactive cells was observed. Furthermore, in some blood group AB individuals, we often encountered the reverse distribution of DBA and GSI-B_4 positive cells, i.e. some clusters of cells reacted with DBA but not with GSI-B_4 and others which were not stained with DBA, reacted with GSI-B_4.
Blood group nonspecific lectins such as PNA, SBA and RCA-I

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tion with neuraminidase(demonstration of sialomucin)

2.Lectin-HRP Staining

Following lectins conjugated with horseradish peroxidase(HRP) were used: Dolichos biflorus agglutinin(DBA), Griffonia simplicifolia agglutinin I-B_4(GSI-B_4), Griffonia simplicifolia agglutinin-II(GS-II), Peanut agglutinin(PNA), Ricinus communis agglutinin-I(RCA-I), soybean agglutinin(SBA) and Ulex europaeus agglutinin-(UEA-I). Blood group ABO and saccharide specificities of these lectins are presented in Table 1. Details of the staining methods have been reported previously(3,4).

Table 1. Lectins used in this study

Lectin	Specificity: Human ABO blood group	Specificity: Sugar	Inhibitory sugar
DBA	A	α-D-GalNAc	D-GalNAc
GSI-B_4	B	α-D-Gal	D-Gal
UEA-I	H(O)	α-L-Fuc	L-Fuc
SBA	None	α/β-D-GalNAc>α/β-D-Gal	D-GalNAc
PNA	None	β-D-Gal(1→3)-D-GalNAc	D-Gal
RCA-I	None	β-D-Gal	D-Gal
GS-II	None	α/β-D-GlcNAc	D-GlcNAc

abbreviations used are GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Gal, Galactose; Fuc, Fucose.

3. Lectin-gold Complex Staining

Methods for preparing colloidal gold with an average particle diameter of 15nm and lectin(DBA,GSI-B_4,UEA-I)-gold complexes were reported previously(6,7,8). Cytochemical staining procedures were performed according to those reported by Nakajima et al(6,7,8).

RESULTS AND DISCUSSION

1. Staining with Histochemical Methods for Mucosubstances

The results are summarized in Table 2. Most of secretory cells in the cervical glands were strongly stained by PAS, moderately by AB pH2.5 and weakly by AB pH1.0. They were stained uniformly dark purple by AB-PAS, but in some cases, the basal region of some secretory cells were stained reddish purple by AB-PAS because of weak staining with AB pH2.5 in these regions. HID

Table 2. Stainability of cervical glands in human uteri with histochemical methods for mucosubstances

PAS	AB(2.5)	AB(1.0)	AB-PAS	HID-AB	AF	Neuraminidase AB-PAS
+3	+1~+2	+1~+2	+2~+3	+1~+3	+1~+2	+2~+3

+1, weak staining; +2, moderate staining; +3, strong staining.

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Mucosubstance and Lectin Histochemistry of Cervical Glands in Human Uteri

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INTRODUCTION

Secretory cells of cervical glands in human uterine cervix are known to secrete various mucous substances including those with blood-group specificity(1). Recent studies have shown that lectins conjugated with horseradish peroxidase or colloidal gold are reliable histochemical reagents for demonstrating blood group antigens and their precursor substances in certain human tissues(2,3,4,5,6,7,8). Although labeled lectins and other histochemical methods for mucous substance have been employed for characterizing mucosubstance in cervical glands(9), these previous studies did not pay attention to the dependence of histochemical results on the blood-group of the tissue donors. The purpose of the present study is to examine properties of the cervical mucins, using various histochemical methods including lectin histochemistry at the light and electron microscopic level.

MATERIALS AND METHODS

Human cervical glands were obtained from 36 biopsy and 6 autopsy cases. Tissues were fixed in 10% formalin and embedded in paraffin, and cut at 4μm. For electron microscopic studies, tissues were fixed in one-half Karnovsky's fixative for 2h at 4°C, dehydrated in a graded series of ethanols, and embedded in L.R.White resin(6,7,8). Blood-group typing of the tissue donors was performed by applying routine hemagglutination test. The secretor status of the tissue donor was determined from the Lewis blood type.

1. Staining with Histochemical Methods for Mucosubstances
(1)periodic acid-Schiff(PAS)(demonstration of all poly-and mucosaccharide-containing hexoses and deoxy hexoses with vic-glycol group);(2)alcian blue pH2.5(AB pH2.5)(demonstration of acidic mucosaccharides, mainly sialomucin);(3)alcian blue pH1.0(AB pH1.0)(demonstration of sulphated mucopolysaccharide); (4)AB pH2.5-PAS double staining(AB-PAS)(demonstration of both neutral and acidic mucopolysaccharide in the same sections); (5)high-iron diamine-AB pH2.5 double staining (HID-AB)(demonstration of both sulphated and other acidic mucopolysaccharide in the same sections);(6)aldehyde fuchsin(AF)(demonstration of sulphated mucopolysaccharides);(7)AB-PAS staining after diges-

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SIMULTANEOUS TYPING OF EsD AND PGM_1 POLYMORPHS IN DENTAL PULPS

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New Delhi and Chandigarh, India

Using starch-agarose medium, eighty symples of blood and human teeth (dental pulp) were subjected to simultaneous typing of Esterase D and $Phosphoglucomutase_1$ enzymes. Three common variants of EsD and PGM_1 were observed and their gene frequencies determined. The results revealed a good agreement between the isoenzymes in dental pulp and blood samples taken from the same subjects. Stability studies showed that the isoenzymes were more stable in dental pulp.

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There have been several polymorphic enzymes occuring in the dental pulp reported earlier; these are PGM_1, PGD, ADA, AK, EsD and Fu polymorphism (Henke et al 1982; Kido et al 1987; Petersen et al 1974; Turowska et al 1977).

In the present study we have demonstrated the presence of groupspecific component (Gc) activity in the dental pulp and added it to the list of enzymes named above. The results show that Gc-subtyping is applicable to bloodstain typing in crime laboratory casework as well as to individual identification. Therefore and due to its high discrimination power (DP = 0,74) Gc-marker is of value concerning forensic investigations.

Acknowledgement. The authors sincerely thank Mrs. M. Prager-Eberle and Mrs. D. Hieronymus for technical assistance.

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Results

Groupspecific ABH-antigen could be determined in the lumen of capillaries filled with erythrocytes. In a few cases positive staining of endothel cells could also be observed. The groupspecific component (Gc) system was detected in dental pulp tissues in all cases tested and its activity was fairly well maintained.

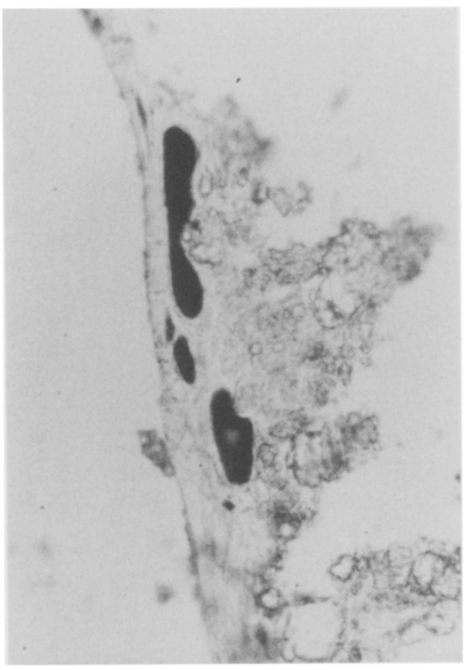

Fig. 1: Cryosection of human dental pulp. ABH-group-specific staining by APAAP procedure.

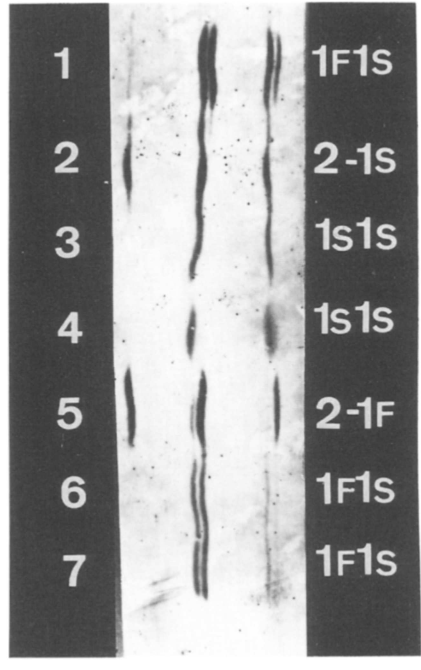

Fig. 2: Gc-subtyping. Isoelectric focusing on LKB-Immobiline Dry plates™ ph 4,5-5,4 and detection with alkaline-phosphatase-linked secondary antibody system.
References: 1,2,5,6,7
Bloodstain: 4
Dental pulp: 3

Discussion

The determination of ABH-bloodgroup in the dental pulp by immunoenzyme technique establishes an essential control of the results obtained by absorption-elution test for confirmation of the ABH-phenotype.

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BLOODTYPING IN HUMAN DENTAL PULP BY IMMUNOENZYME TECHNIQUES

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Introduction

Genetically determined markers increase the chances of individual identification of human cadavers, especially in cases in which tests cannot be made by direct methods because of detoriation, in putrefied and skeletonized cadavers.

The ABH-system is important, because it appears to be more stable concerning autolytic processes than other antigens and is usually documented of most persons. ABH-bloodtyping in human teeth and dental pulps by absorption-elution test is possible but can easily lead to false results (von Crainic et al 1981).

The extensive vascularity of the human dental pulp and its protection by dentine offers excellent possibilities for bloodtyping by application of immunoenzyme techniques.

Material and Method

Studies were made of 24 extracted molar teeth after 10 to 18 month storage at room temperature. After cutting the tooth longitudinally the pulp was separated from the dentine. Tissue sections were made using a cryostat at -20°C and from formalin fixed and paraffin embedded material. Immunoenzyme techniques as peroxidase-anti-peroxidase (PAP) method and alkaline-anti-alkaline phophatase (APAAP) method were applied for ABH-bloodgrouping as described by Bourne 1983.

Isoelectric focusing was performed essentially by the method of Pflug (1986) using LKB-Immobiline Dry Plates ™pH 4,5-5,4 followed by alkaline phosphatase-linked secondary antibody immunoenzyme technique staining system.

Materials: DaKo-Universal-Mäuse-PAP$_{Kit}$(K 550), Universal-DaKo-APAAP-Kit$_{TM}$-Maus (K 670), Monoclonal Anti A (A 581), Monoclonal Anti B (A 582), Mäuseserum (X 910), Schweineserum (X 901), Glycergel Einschlußmedium (C 563) DAKOPATTS, Hamburg, FRG. LKB Immobiline Dry Plates ™pH 4,5-5,4 (Pharmacia LKB, Freiburg, FRG). Goat-anti Human Gcglobulin-IgG fraction (ATAB, Scarborough, USA). Anti-Ziege IgG alkalische Phosphatase markiert, 5-Brom-4-chlor-3-Indolylphosphat (Sigma Chemie, München, FRG).

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gekehrt kann es vorkommen, dass Blutflecken von einer Geburt relativ wenig absorbieren; bei Berücksichtigung, dass Blute von Schwangeren eine rund 3-5 mal geringere Enzymaktivität aufweisen als Geburtsblutspuren (*Oya et al.1973, Oya 1985*), fallen diese in einen Absorptionsbereich, in welchem immer mit dem Vorliegen von Menstruationsblut gerechnet werden muss, und in welchem auch Mischspuren aus Vaginal(-Sperma)sekret und Blut absorbieren. *Der Aktivitätsnachweis von hitzestabiler alkalischer Phosphatase in Blutspuren lässt somit verschiedene Interpretationsmöglichkeiten zu.* Aus einem positiven Aktivitätsnachweis darf jedenfalls nicht (wie bei *Oya et al.1973, Oya 1985, Oepen und Köhler 1977*) vorbehaltlos (Ausnahme Regan-Enzym) auf Schwangeren-/Geburtsblut geschlossen werden. Welche der oben angeführten Interpretationsmöglichkeiten im Einzelfall die richtige ist, kann nur über weiterführende Untersuchungen zu klären versucht werden.

So haben wir zur Beurteilung des Absorptionsergebnisses des eingangs beschriebenen Falles sowie an Spurstichproben von Menstruationsbluten von 23 Frauen einen Schwangerschaftstest durchgeführt (ORTHO BETA-20 EIA MONOKLONAL). In keinem Ansatz liess sich beta-HCG (Human Chorionic Gonadotropin) nachweisen; im Gegensatz dazu war in allen Stichproben der Geburtsblute beta-HCG Schwangerschafts-indizierend vorhanden. Für späte Schwangerschaftsstadien dürfte sich auch der Nachweis von HPL (Human Placental Lactogen) eignen.
Da keine der untersuchten Blutspuren aus der Fingerbeere der Frauen, von welchen wir Menstruationsblut erhalten hatten, sich durch eine relevant stärkere Absorption vom männlichen Probandenblut unterschied, kann eine fotometrische Bestimmung der hAP im Venenblut als weiteres Kriterium herangezogen werden, ob eine Blutspur nun tatsächlich von einer menstruierenden Frau verursacht worden sein kann bzw. allenfalls von einer Frau stammen könnte, die beim Geschlechtsverkehr verletzt wurde; basierend auf den Befunden von *Oya* sowie *Köhler* müsste nämlich Blut von Schwangeren im Gegensatz dazu eine Enzymreaktion aufweisen.

Im eingangs geschilderten Fall bestätigten unsere Befunde (hAP, Schwangerschaftstest sowie weitere Untersuchungen) die Aussage der ermittelten Spurenverursacherin: überraschend starkes Einsetzen der Menstruation nach einem Geschlechtsverkehr.

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Für die Mithilfe bei der Beschaffung von Menstruationsblutproben danken wir Frau B. Furrer, für die Asservierung von Geburtsblutspuren Herrn OA Dr. U. Herrmann, Universitäts-Frauenklinik Bern.

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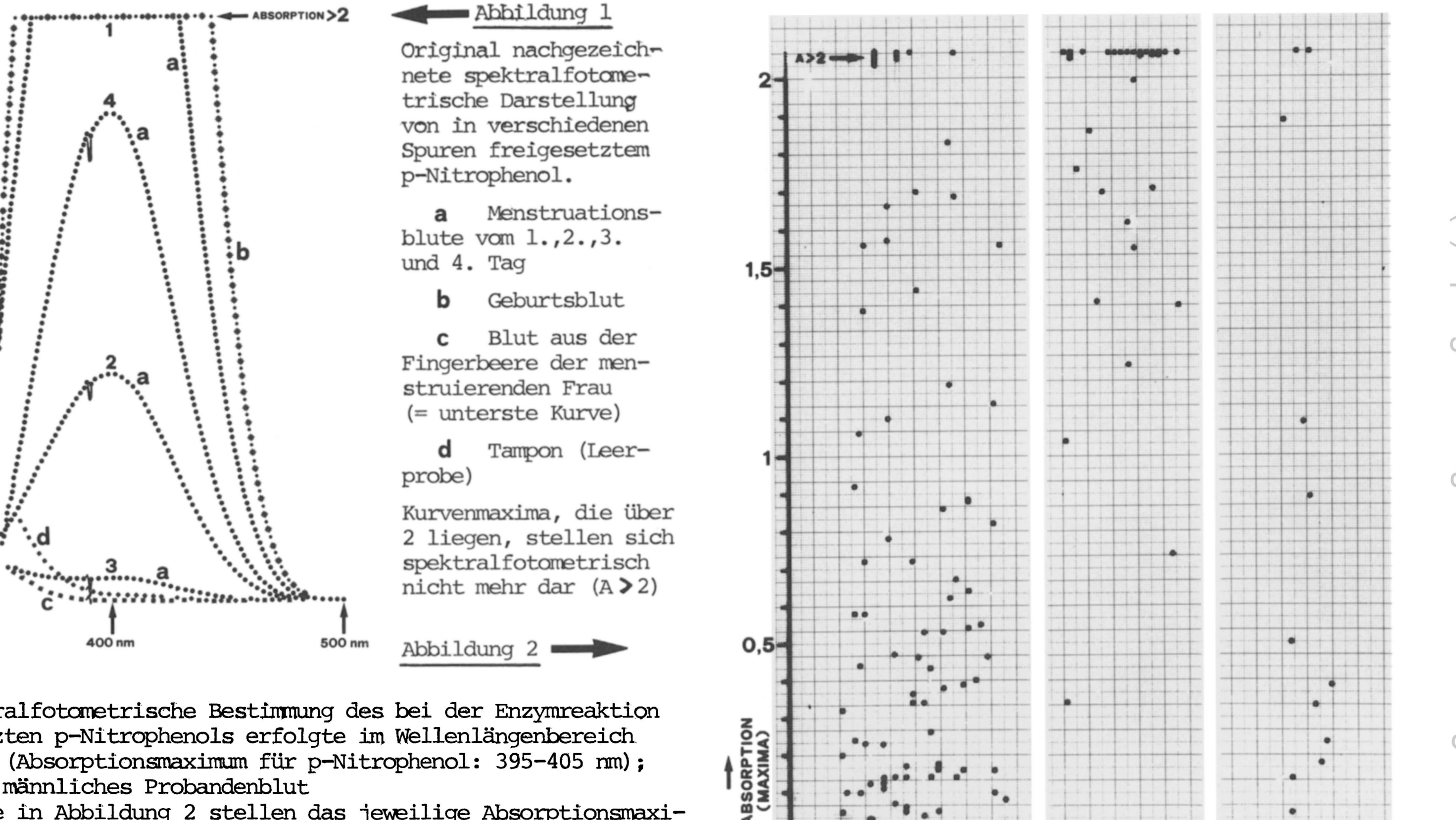

Abbildung 1

Original nachgezeichnete spektralfotometrische Darstellung von in verschiedenen Spuren freigesetztem p-Nitrophenol.

a Menstruationsblute vom 1.,2.,3. und 4. Tag

b Geburtsblut

c Blut aus der Fingerbeere der menstruierenden Frau (= unterste Kurve)

d Tampon (Leerprobe)

Kurvenmaxima, die über 2 liegen, stellen sich spektralfotometrisch nicht mehr dar (A > 2)

Abbildung 2

Die spektralfotometrische Bestimmung des bei der Enzymreaktion freigesetzten p-Nitrophenols erfolgte im Wellenlängenbereich 350-500nm (Absorptionsmaximum für p-Nitrophenol: 395-405 nm); Leerwert: männliches Probandenblut

Die Punkte in Abbildung 2 stellen das jeweilige Absorptionsmaximum der einzelnen Spektren dar. Bei den genau übereinander liegenden Punkten handelt es sich um Spurstichproben von zu verschiedenen Zeitpunkten gewonnenen Asservaten derselben Frau (z.B. Tampons vom 1.,2.,3. und 4. Tag der Menstruation). Kurvenmaxima mit einem höheren Absorptionswert als 2 sind am oberen Bildrand mit dem Vermerk A > 2 dargestellt.

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und dass dies auch bei der Verabreichung von Progesteron-haltigen Präparaten der Fall sei.

Ohne näher darauf einzugehen, inwieweit die Untersuchungen von *Smith et al.* (1970) im einzelnen von anderen Autoren bestätigt wurden, erschien es uns angebracht, neben Menstruationsblutproben und Blutspuren von Frauen, die geboren hatten, zur Kontrolle auch einige unblutige Vaginalabstriche sowie Spermaspuren aus Notzuchtdelikten mitzuführen.

METHODEN

Material

- 91 Menstruationsblutproben (Tampons, Binden, Wäsche) von 28 Frauen
- Auf Baumwollstoff aufgetropftes Blut aus einer Fingerbeere von 23 der 28 Frauen
- 32 mit Blut befleckte Binden von 19 Frauen, die eben erst oder am Tag zuvor geboren hatten
- 8 Vaginalabstriche und 4 (mit Vaginalsekret vermengte) Spermaspuren (aus Notzuchtsachen; Schwangerschaftstest soweit bekannt negativ)
- Auf Baumwollstoff aufgetropftes Kapillarblut von männlichen Probanden (Leerwerte).

Fotometrischer Aktivitätsnachweis der hAP

Der Aktivitätsnachweis erfolgte mittels der von *Oepen und Köhler* (1977) modifizierten Technik nach *Oya, Asano und Fuwa* (1973):

1. 3×1 cm^2 (oder weniger) Spuren-/Kontrollmaterial ausschneiden und zerstückelt mit Carbonat-Puffer pH 10.05 nach *Delory und King* (1945) bedecken.
   Puffer: 0.1 M Na_2CO_3 - 0.1 M $NaHCO_3$ - 1:1
2. Eliminieren der hitzelabilen AP; Inkubieren der Ansätze: 40 Min. bei 60°C.
   Anmerkung: In der Vorschrift von *Oepen und Köhler* (1977) wird den ausgeschnittenen Probenstückchen 0,5 ml Puffer zugegeben. Wegen der starken Saugfähigkeit der Tampons/Binden erhöhten wir die Menge zugegebenen Puffers in der Weise, dass nach dem Eliminieren der hitzelabilen AP 0,5 ml Lösung herauspipettiert und in ein neues Röhrchen übertragen werden konnten.
3. 0,5 ml Substratlösung zugeben und die Ansätze während 24 Std. bei 37°C zur Enzymreaktion inkubieren. Substratlösung: 0,443 g 4-Nitrophenylphosphat Dinatriumsalz - MERCK Nr. 6850) in 100 ml 0.001 N HCl.
4. Unterbrechen der Enzymreaktion mit 1 ml 10%-iger Trichloressigsäure.
5. Ansätze durch Faltenfilter (S&S 595 1/2) filtrieren.
6. 0,5 ml Filtrat (klar, farblos) mit 2 ml 0.2 N NaOH alkalisieren [Gelbfärbung bei positiver Enzymreaktion (Substratspaltprodukt: p-Nitrophenol].
7. Absorptionsmessung gegen Leerwert (Absorptionsmaximum für p-Nitrophenol: 395-405 nm)
   Leerwertansätze (Reihenfolge des Vorgehens): 1./2./4./0,5 ml Substratlösung/ 5./6./7. Leerwertersatz: männliches Probandenblut (Ansatz wie Spuren, 1.-7.)

ERGEBNISSE UND DISKUSSION

Die Ergebnisse sind in Abb. 1 und Abb. 2 dargestellt.

Wesentlicher Befund unserer Untersuchungen ist, dass selbst ein hohes Absorptionsmaximum beim fotometrischen Aktivitätsnachweis von hAP im Einzelfall das Vorliegen von Blut einer Schwangeren oder von Blut, das die Geburtswege passiert hat, *nicht beweist*, sondern allenfalls als Hinweis hierfür interpretiert werden darf. Auch *Menstruationsblute* können einen sehr hohen Gehalt an aktivem Enzym aufweisen, und dies kann durchaus auch für *blutige Vaginalabstriche* einer Frau zutreffen, die z.B. bei einem Geschlechtsverkehr *verletzt* wurde. Um-

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Zum Beweiswert des Schwangerschaftsnachweises an Blutspuren mittels fotometrischer Aktivitätsbestimmung der hitzestabilen alkalischen Phosphatase (hAP)

Reliability of the forensic diagnosis of pregnancy from bloodstains by photometric determination of the heat-stable alkaline phosphatase activity

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ZUSAMMENFASSUNG: *Der Nachweis von hitzestabiler alkalischer Phosphatase in einer Blutspur ist für das Vorliegen von Schwangerenblut und/oder Geburtsblut nicht beweisend. Es lässt sich im Einzelfall nicht ausschliessen, dass es sich dabei auch um Menstruationsblut oder um mit vaginalen Ausscheidungen vermengtes Verletzungsblut handeln kann.*

SUMMARY: *The presence of heat-stable alkaline phosphatase activity in blood stains cannot be considered to be indicative of venous blood of a woman in pregnancy and/or of blood from parturition. It cannot be excluded that such a blood stain could also originate from menstrual discharge or from blood that became mixed with vaginal secretions.*

EINLEITUNG

Auf einem öffentlichen Parkplatz eines Schweizer Ferienortes waren eine grosse Blutlache festgestellt und in der Umgebung ein blutiges Stoffstück sowie im Mülleimer einer Toilette ein durchbluteter Damenslip sichergestellt worden. Wir erhielten die Asservate von der Polizei um abzuklären, ob das Blut eventuell von einer Schwangeren bzw. von einer Geburt stammen konnte.

Im Rahmen der Untersuchungen führten wir an den Blutasservaten u.a. einen fotometrischen Nachweis zur Aktivität von hitzestabiler alkalischer Phosphatase durch, hatten doch *Oepen und Köhler (1977)* darauf hingewiesen, dass *die von ihnen modifizierte Methode von Oya, Asano und Fuwa (1973) eine sichere Unterscheidung von venösem Schwangeren- und Nichtschwangerenblut erlaube, und dass dies auch für Plazentablut gelte.*

Zur Kontrolle wurden von uns noch Menstruationsblutspuren zur Enzymreaktion angesetzt. Da sowohl in den polizeilichen Blutasservaten als auch in den Menstruationsblutspuren eine Enzymaktivität festgestellt werden konnte, führten wir zur Ueberprüfung dieses Befundes nunmehr an zahlreichen eingetrockneten Menstruationsblutproben eine fotometrische Bestimmung von durch hitzestabile alkalische Phosphatase freigesetztem p-Nitrophenol durch.

Eine im Menstruationsblut im Vergleich zu Blut aus dem Kreislauf erhöhte Enzymaktivität hatten bereits *Smith, Hunter und Spadoni (1970)* beschrieben, wobei allerdings nicht unterschieden wurde zwischen hitzestabiler und -labiler Fraktion des Enzyms. Dieselben Autoren wiesen - wie andere auch - ausserdem darauf hin, dass der Anteil an alkalischer Phosphatase in Geweben/Sekreten des weiblichen Genitaltraktes zyklusabhängig sei; sie stellten darüber hinaus in eigenen Untersuchungen fest, dass es bei der Behandlung von Patientinnen mit Gonadotropin zu einer starken Erhöhung der Enzymaktivität in der Cervixschleimhaut kommt,

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swab has been collected 8 h after the rape on a woman alive; there was certainly no seminal plasma left. Note that acid phosphatase tests were also negative.

(4) The semen material was very small because it had been used for the analyses required by the court during judicial proceedings.

(5) It is perhaps an old semen stain which was cleaned. It may be presumed that spermatozoa persisted (Cordonnier 1922) whereas the seminal fluid itself did not.This fact is surprising but it has been reproduced experimentally in our laboratory.

CONCLUSION

The detection of seminal P 30 glycoprotein by ELISA testing is QUICK (results are obtained within 24 h with a short effective working time), EASY (large scale analyses till 30 tests a day with one technician), REPRODUCIBLE (variation coefficient : 12 %), SENSITIVE (threshold of P 30 detection : 1.5 ng/mL), SPECIFIC (no cross reaction observed with other human biological fluids or secretions).

In short, this test should be regarded as a very helpful complementary analysis to spermatozoa research in forensic expert cases. It shows better specificity and sensitivity than seminal acid phosphatase testing. We now apply it routinely in our laboratory.

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Table 1. Results are presented separately for vaginal swabs, other swabs and stains on clothes, towels a.s.o..
The P 30 results are expressed by the last positive dilution.
SPZ = spermatozoa and AP = acid phosphatase.

20 vaginal swabs

REF.	AGE	SPZ	P30	AP
ML1158	7 y	+++	1/4	+++
T 236	3 y	++++	1/256	+++
T 259	2.5 y	-	-	-
T 369	1 y	++++	1/32	+
T 385	7 m	+++	1/32	+
T 399†	7 m	-	-	-
T 482	6 m	-	-	NT
T 419	5 m	+	1/1	+++
T 414	5 m	+++	1/256	+++
T 426	4 m	++	1/32	+++
T 433†	3 m	-	1/16	+(1)
T 440	3 m	+++	1/32	+++
T 441	3 m	-	-	++
		-	-	-
T 457	1 m	+	1/256	-
T 461	1 m	++++	1/1024	NT
T 462	2 w	-	-	++
		-	-	NT
T 496	1 w	++	1/16	NT
T 470	2 d	-	-	NT

†dead body

5 anal swabs
2 buccal swabs

REF.	AGE	SWAB	SPZ	P30	AP
ML1158	7 y	anal	+	-	-(2)
		buc.	+	-	-(3)
T 94†	5 y	anal	-	-	-
T 207	3 y	anal	-	-	-
T 399†	7 m	anal	-	-	-
		buc.	-	-	-
T 434	5 m	anal	-	-	-

†dead body

47 diverse clothes, towels and so one

REF.	AGE	TYPE OF STAINS	SPZ	P30	AP
ML 972	10 y	preservatives	+++	1/4	-
		slip	-	-	NT
ML1158	7 y	menstrual strip	+++	1/8	+++
T 40	6 y	slip	+	1/32	+++
T 61	5 y	slip	-	-	-
T 73	5 y	pyjama	+++	1/64	+++
T 83	5 y	kleenex	+++	1/32	+++
T 120	4.5y	dress	+++	1/16	+++
T 102	4.5y	handkerchief	+	1/2	+++
		handkerchief	+	-	- (4)
		slip	+++	1/32	+++
T 143	4 y	slip	-	-	-
T 170	3.5y	slip	+++	1/64	+++
T 173	3.5y	slip	-	-	NT
T 207	3 y	handkerchief	+++	1/32	+++
		slip	+	1/2	-
T 209	3 y	trousers, slip	-	-	-
T 236	3 y	handk.,wrapper	++++	1/256	+++
T 220	2.5y	slip	+++	1/16	+++
T 233	2.5y	slip	+++	1/128	+++
T 232	2.5y	bust-bodice	+++	1/64	+++
		slip	-	-	-
T 240	2.5y	slip	++	1/128	+++
		kleenex	+	1/8	++
		glove	++	1/32	+++
T 286	2 y	towel	+++	1/1024	+++
T 340	1.5y	slips,handk.	-	-	-
T 347	1 y	slip	-	-	-
T 369	1 y	slip	+	1/16	-
T 376	1 y	slip	-	-	-
T 487	1 m	slip	-	-	NT
		kleenex	-	1/8	NT(1)
T 462	2 w	prostitute's clothing	+	-	++(5)
		slips	-	-	NT
T 466	1 w	mantle	-	-	NT
T 496	1 w	slip	++	1/64	NT
		kleenexes	+++	1/256	NT
T 470	2 d	towel	+++	1/128	NT

(3) According to Enos (1978) : " it is possible to identify spermatozoa in oral smears up to 6 hours after the attack, despite brushing teeth and drinking". In this case, buccal

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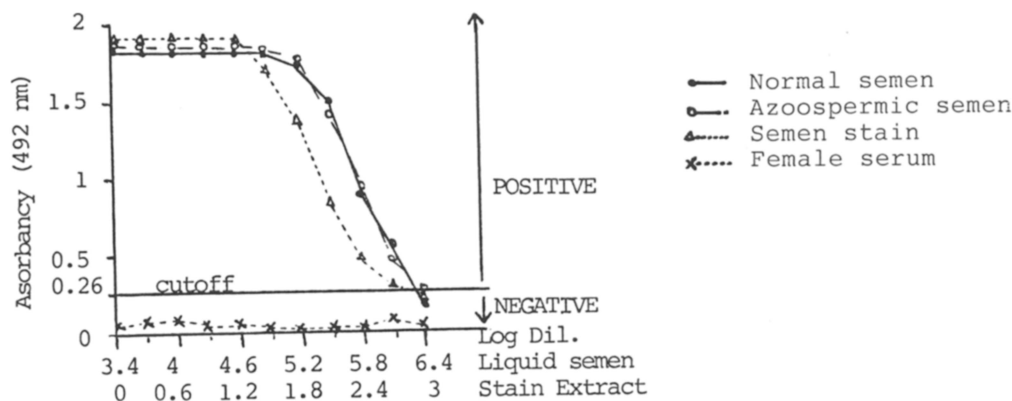

Fig. 1. P 30 detection (ordinate = absorbency at 492 nm) as a function of dilutions (abscissae = dil. log.)

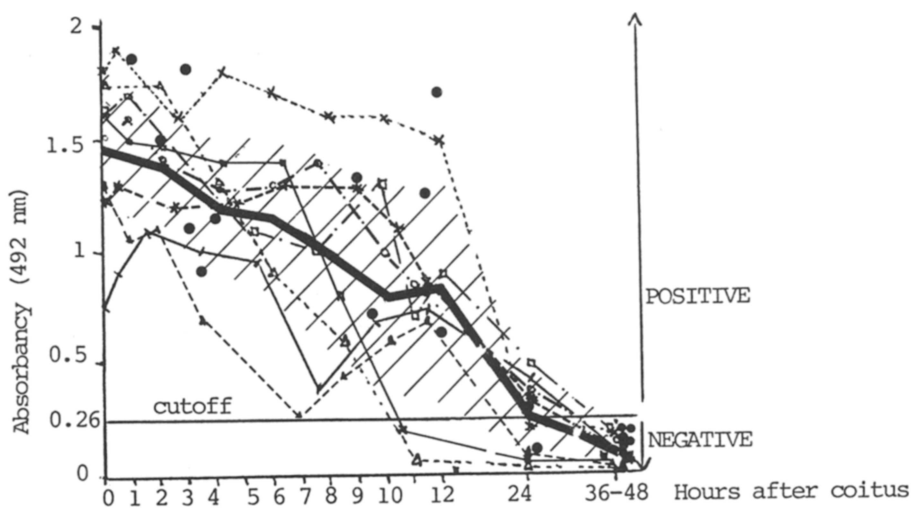

Fig. 2. P 3O detection in vagina (ordinate = absorbency at 492 nm) as a function of time after coitus (absissae = hours).
● : vaginal swabs from the 19 women (a)
eight curves : decay for each volunteer donor (b)
heavy line : mean decay for the 99 swabs (a and b)
hachured area : value of + and - one σ

Six discrepancies have been observed that need some comments :

(1) possible azoospermic semen ? . The presence of semen is in agreement with the history. (According to Willot (1982) the frequency of azoospermia is 1.9 % in sexual assault cases).

(2) Possible false positive result due to artefacts or contamination from the vaginal contents (Enos 1978).

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$NaHCO_3$ 0.1 M pH9.5). Plates were then washed three times with PBS-BSA-TWEEN buffer. After drying, they were ready to receive the samples to be tested. 50 μL of sample in twofold dilutions in PBS-BSA-TWEEN buffer were kept 2 h at 37° C; after three further washings, 50 μL of the optimal dilution (in PBS-PSA-TWEEN buffer 1/8000) of mouse anti P 30 monoclonal antibody (Hybritech) were added to each well and incubated for 2 h at 37°C. After three further washings, fixation of the monoclonal antibody was revealed by using 50 μL of Peroxydase-conjugated rabbit Ig anti-mouse Ig (Dako) at 1/250 dilution. This reagent was left for 1 h at 37°C. and the excess removed by three washings with running water. 50 μL of substrate solution (OPD) were added to each well and the reaction immediately stopped by adding 150 μL 2N H_2SO_4. An orange coloration indicated positive samples whereas negative samples remained colorless . Absorbency values at 492 mm were measured by using a spectrophotometer (Multiskan, Titertek). The results are expressed in terms of the last dilution of the samples that still gives a significant absorbency value.

RESULTS

Cutoff Value : 11 whole blood, 14 sera, 9 saliva, 3 perspirations, 5 nasal secretions, 13 urina, 14 feces , 12 vaginal secretions and 2 menstrual blood samples were tested; they manifested no P 30 antigenic activity. The cutoff of the test calculated as the (mean + 2σ) of these negative controls was found to be equal to 0.26.

Variation Coefficient : The calculated variation coefficient ($CV = 100 \frac{\sigma}{m}$) is 12 % for the absorbency of 1. (N Replicate = 9)

Experimental Study : Fifteen different liquid sperm samples were analyzed (among them 1 vasectomized, 2 medical azoospermic and 2 oligozoospermic). The last detectable dilution was around 1/1,000,000. Consequently the detection limit -estimated from the mean level of P 30 in normal sperm- is 1.5 ng/mL. Fifty-four experimental stains from different sperm samples have been analyzed (see Fig. 1).

Conservation in Vagina : 19 vaginal swabs (a) were collected from 19 women and the interval time after coitus is known. 80 vaginal swabs (b) were collected from 8 volunteer donors; each woman collected herself 10 vaginal swabs at different time intervals after coitus. Figure 2 shows the decay curves of P 30 detection in vagina as a function of time.The mean delay for P 30 detection is 24 h with a range from 10 1/2 h to 24 h.

Application to Forensic Caseworks : 98 analyses were carried out on 74 exhibits from 42 forensic cases (dating from 2 days to 10 years) and were compared with spermatozoa and acid phosphatase researches. The results are given in table 1.The sensitivity and the specificity of P 30 test compared to spermatozoa test are respectively 92.7 % and 95.3 %.

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P 30 and rape

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INTRODUCTION

The detection of sperm in swabs or stains is usually performed by using two methods : search for spermatozoa and for seminal acid phosphatase.The presence of spermatozoa provides valuable evidence except when the raper is azoospermic. Nevertheless, it is not a practical test for large scale screening because of ocular weariness due to microscopic examination. Furthermore, even with considerable experience, it is difficult to recognize morphologically abnormal spermatozoa. With regard to the second test, seminal acid phosphatase, the problem is that vaginal secretions may sometimes give false positive reactions.

For all these reasons, a really specific, easy and sensitive marker of human seminal plasma would be of great interest to forensic medicine.A prostate specific glycoprotein called P 30 has been described in the literature; its molecular weight is about 30,000 d (Sensabaugh 1978) and its concentration in normal semen plasma has a mean level of 1.55 mg/mL (Graves 1985). The aim of the present study is to perfect an ELISA test for sensitive P 30 detection, and to apply it to sexual assault evidence.

MATERIALS AND METHODS

Samples : Semen samples were obtained from the Urology Clinic. Vaginal secretions samples - collected with sterile cotton wool swabs - were obtained from the Gynecology Clinic and from volunteer donors as well. Forensic expert cases, from 1977 to the present, were included in the trial when the remaining materials (suspect stains and swabs) were available in sufficient amounts. Data concerning spermatozoa (always) and acid phosphatase (sometimes) analyses were obtained as required at the time of facts.

Extraction : A 5 mm long thread of stain or 4 mm^2 of swab were extracted overnight at 4° or 20° C in 250 μL of PBS-BSA-TWEEN buffer ($NaH_2PO_4.H_2O$ 0.52 g; $K_2HPO_4 3H_2O$ 3.67 g; NaCl 8.76 g; H_2O 10 L containing 0.3 % bovine serum albumine and 0.1 % Tween 20).

ELISA test : Polystyrene immunoplates (CEB) were coated overnight at 4° C. with 75 μl per well of rabbit Ig anti P 30 (DAKO) diluted (1/8000) in carbonate buffer (Na_2CO_3 0.1 M;

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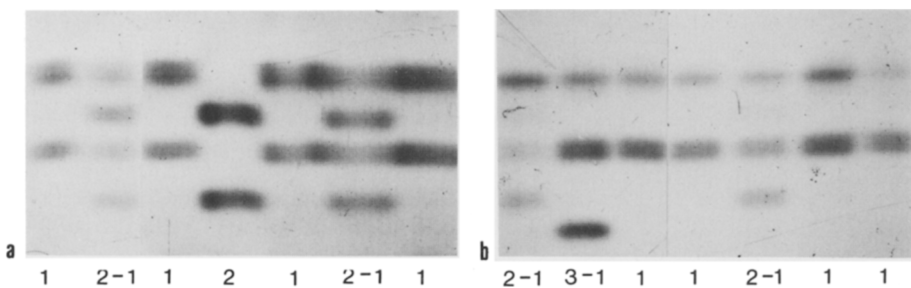

Fig. 2. DIA3 types in fresh dental pulps (a) and fresh hair roots (b). The anode is at the top

Der DIA3-Nachweis gelang auch an bis zu 2 Wochen bei Zimmertemperatur gelagerten Zahnpulpen und an frischen Haarwurzeln (Fig. 2).

SUMMARY

Polymorphism of DIA3 was investigated by isoelectric focusing in semen samples from 235 unrelated Japanese volunteers and patients. Besides the three common phenotypes seven samples of the type 3-1 were observed. However, readable isoenzyme patterns were not demonstrated in semen samples of oligospermia under about 10×10^6 /ml sperm cells. The allele frequencies were DIA3*1 = 0.821, DIA3*2 = 0.164 and DIA3*3 = 0.015. The DIA3*1 frequency in oligospermia (0.765) was lower than that in normospermia (0.836). DIA3 types were determined from seminal stains stored at 37°C for up to 4 weeks, at room temperature for up to 8 weeks and at 4°C for over 12 weeks. In vaginal swabs the isoenzyme bands were very faint and not identifiable. DIA3 phenotyping was also possible from dental pulps stored at room temperature for up to 2 weeks and from fresh hair roots.

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Table 1. Distribution of DIA3 types in a Japanese population

Phenotype	No. observed				No. expected
	Normo-spermia[a]	Oligo-spermia[b]	Total[c]	(%)	
1	129	27	156	(66.4)	158.4
2-1	47	20	67	(28.5)	63.3
2	4	1	5	(2.1)	6.3
3-1	6	1	7	(3.0)	5.8
3-2	0	0	0	(0.0)	1.2
3	0	0	0	(0.0)	0.1
Total	186	49	235	(100.0)	235.1

[a] DIA3*1 = 0.836, DIA3*2 = 0.148, DIA3*3 = 0.016; χ^2 = 1.132, d.f. = 3, 0.8 > P > 0.7

[b] DIA3*1 = 0.765, DIA3*2 = 0.225, DIA3*3 = 0.010; χ^2 = 1.889, d.f. = 3, 0.7 > P > 0.5

[c] DIA3*1 = 0.821, DIA3*2 = 0.164, DIA3*3 = 0.015; χ^2 = 2.068, d.f. = 3, 0.7 > P > 0.5

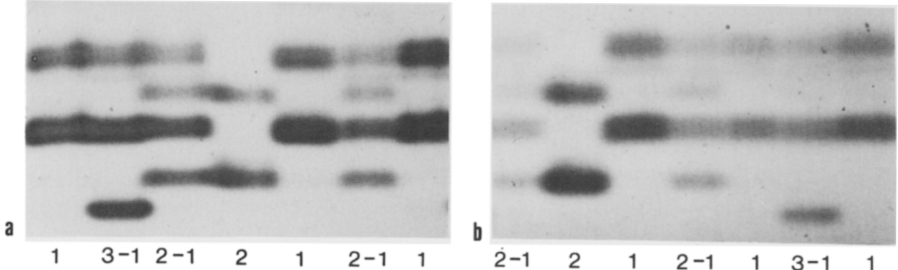

Fig. 1. DIA3 types in fresh semen samples (a) and seminal stains stored at room temperature for 4 weeks (b). The anode is at the top

Die Isoelektrofokussierungsmethode wurde mit Erfolg zur DIA3-Typisierung an Spermaspuren angewandt; die betreffenden Phänotypen konnten bei 37°C bis zu 4 Wochen, bei Zimmertemperatur bis zu 8 Wochen und bei 4°C mehr als 12 Wochen nach Beginn der Lagerung nachgewiesen werden. Die ermittelte zeitliche Nachweisgrenze von 8 Wochen bei Zimmertemperaturlagerung ist offensichtlich länger als die in der Stärkegelelektrophorese feststellbare Nachweisgrenze von 4 Wochen (Oepen et al. 1980). Die hier angegebene Isoelektrofokussierungsmethode ist daher zur DIA3-Typisierung aus Spermaspuren besser geeignet. Fig. 1b zeigt das Muster der DIA3-Typen an 4 Wochen bei Zimmertemperatur gelagerten Spermaspuren.

An Scheidenabstrichen waren die Isoenzymbanden sehr schwach und nicht erkennbar.

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Zahnpulpen

Zähne wurden 53 Patienten gezogen, die an der Zahnklinik der Medizinischen Universität Yamanashi behandelt wurden. Das Zahnbein wurde mit einem Hammer zerschlagen und die Zahnpulpa vom Pulpencavum entnommen. Das Pulpengewebe (10-20 mg) wurde auf einer Hohlglasplatte in 0,01 ml 1 % Triton X-100 eingeweicht und mit einem Glastab zerquetscht.

Haarwurzeln

Kopfhaare wurden 25 Lebenden ausgezogen. Fünf bis zehn Haarwurzeln mit Wurzelscheidezellen wurden auf einer Hohlglasplatte in einem Tropfen von 1 % Triton X-100 eingeweicht und mit einem Glastab zerrieben.

Isoelektrofokussierung

Gerät: LKB 2117 Multiphor
Gelgröße: 230×110×0,5 mm
Gelzusammensetzung:

5,25 % Acrylamid/0,25 % Bisacrylamid	20 ml
Ampholine pH 3,5-10 (LKB)	0,33 ml
Ampholine pH 4-6 (LKB)	0,33 ml
Ampholine pH 6-8 (LKB)	0,33 ml
0,01 % Riboflavin	0,3 ml
Saccharose	2,5 g

Anodenpuffer: 1 M Phosphorsäure
Kathodenpuffer: 1 M Natriumhydroxid
Probenauftrag: mit 5×6 mm Filterpapierplättchen (Toyoroshi Nr. 2, Tokio) 2 cm vom anodalen Gelrand entfernt
Lauf: 200 bis 1400 V für 60 min, alle 10 min um 200 V stufenweise steigern, anschließend 1400 V für 120 min

Färbung

Agarose-Overlay-Technik nach Kühnl et al. (1977)
Substrat: Dichlorphenol-Indophenol
Coenzym: NADH für Sperma, Scheidenabstriche und Haarwurzeln
NADPH für Zahnpulpen

ERGEBNISSE UND DISKUSSION

Die Verteilung der DIA3-Typen in der untersuchten Population ist in Table 1 zusammengestellt. Neben den drei häufigen Phänotypen wurden sieben Proben des Typs 3-1 beobachtet (Fig. 1a). Entgegen den Erwartungen von Caldwell et al. (1976) und von Kühnl et al. (1977) war die DIA3*1-Frequenz bei Oligospermie mit Spermienzahlen von $10\text{-}40 \times 10^6$/ml niedriger als diejenige bei Normospermie. Dieser Unterschied dürfte auf eine kleine Populationsgröße für Oligospermie zurückgeführt werden. Bei hochgradiger Oligospermie unter etwa 10×10^6/ml Spermien ließen sich jedoch keine ablesbaren Isoenzymbanden nachweisen.

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DIA3-Typisierung an menschlichen Spermaproben, Spermaspuren, Zahnpulpen und Haarwurzeln mittels Isoelektrofokussierung

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EINLEITUNG

Der Polymorphismus der menschlichen spermaspezifischen Diaphorase wurde erstmals von Caldwell et al. (1976) beschrieben und wenig später von Fisher et al. (1977) als DIA3 bezeichnet. Es lassen sich sechs häufige Phänotypen elektrophoretisch unterscheiden, die von drei kodominanten Allelen, DIA3*1, DIA3*2 und DIA3*3, gesteuert werden (Kühnl et al. 1977).

In der vorliegenden Arbeit haben wir die Verteilung der DIA3-Typen in einer japanischen Bevölkerung mit Hilfe einer verbesserten Isoelektrofokussierungstechnik untersucht. Ferner haben wir versucht, dieses Enzym an menschlichen Spermaspuren, Zahnpulpen und Haarwurzeln nachzuweisen.

MATERIAL UND METHODE

Spermaproben

Ejakulate wurden von 96 in Yamanashi-ken lebenden, gesunden Spendern und 156 Patienten der Fertilitätssprechstunde des Zentralen Krankenhauses Yamanashi-ken gewonnen.

Spermaspuren

20 Ejakulate mit bekannten Phänotypen wurden auf Filterpapier (Toyoroshi Nr. 2, Tokio) getrocknet und bei 37°C, Zimmertemperatur und 4°C unterschiedlich lange (bis zu 12 Wochen) gelagert. Diese Spuren wurden in 5×6 mm Plättchen geschnitten, mit wenig Aqua dest. angefeuchtet und untersucht.

Scheidenabstriche

Von 30 Frauen, die seit 1 Woche keinen Geschlechtsverkehr gehabt hatten, wurden anläßlich einer Untersuchung an der Frauenklinik des Zentralen Krankenhauses Yamanashi-ken Scheidenabstriche mit Wattentupfern entnommen. Die äußere sekretreiche Schicht der Watte wurde vom Stäbchen abgezogen, in 5×6 mm große Stücke zerschnitten, mit wenig Aqua dest. angefeuchtet und untersucht.

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DISCUSSION

Isoelectric focusing is a sensitive method but limitated by frequent distortions in the PGM pattern of semen and vaginal samples, due to their high and variable salt content. Dialysis of microsamples is not convenient. In order to be informed of a possible distortion, non polymorphic bands were stained with Coomassie blue, in the upper part of the gel where the locus 2 PGM bands are usually absent.

Seminal PGM is a highly polymorphic and stable protein. Neverthe-less, results obtained in casework are disappointing, due to the complex situation in body fluid mixtures.

The success rate of seminal PGM typing in vaginal swabs is low, for different reasons: 1) the rapid elimination of seminal PGM, 2) the high vaginal PGM activity possibly masking seminal PGM and 3) the frequent occurrence of smears in isoelectric focusing patterns. Better results should be obtained from vaginal drainage stains and, when possible, even more from other semen stains.

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Stability of Semen PGM in the Vagina after Sexual Intercourse

Eight couples provided a timed intervals set of post-coital vaginal swabs and a pre-coital semen-free swab as well. The swabs, immediately dried were analyzed within the next few days. Donors n° 4, 8 and 9 remained lying for eight hours after intercourse whereas the others were active. The results, summarized in the table, show that vaginal PGM activity was increased whereas seminal PGM subtypes could only be determined during a short period (seminal PGM was still typable after 6.5 h in donor n° 9). These findings are in agreement with those previously reported (Price et al. 1976; Eastwood 1977; Davies 1982; Garlo 1985). The PGM are optimally typable few minutes after intercourse. After 30 min., the enhancement of PGM activity often causes smears on the gels, affecting the bands'resolution. PGM activity presents individual variations. This could be partially due to the manner of collection of the samples, that were taken by the donors themselves. PGM activity was generally found to be associated to high amounts of semen as reflected by high P30 concentration..

Table 1: PGM subtypes in semen-positive vaginal swabs

donor	test	blood female	blood semen male	time after intercourse (h)							
				prior	0.1	0.5	1.5-2.5	3.5-6.5	5.5-6.5	7.5-8.5	9-10
2)	PGM	2B1B	1A1B	neg	1A1B	(1A) (1B)	1A1B	neg	neg	neg	
	P30			neg	$>2^{-10}$	2^{-4}	$>2^{-10}$	2^{-6}	2^{-4}	2^{-3}	
3)	PGM	2A1B	1A	2A(1B)	2A(1B) 1A	2A1B1A	2A1B1A	2A(1B) (1A)	neg	neg	
	P30			neg	$>2^{-10}$	$>2^{-10}$	$>2^{-10}$	$>2^{-10}$	2^{-7}	2^{-6}	
6)	PGM	1A1B	2B1B	neg	(1A) (1B) (2B)	1A1B2B	1A1B2B	neg	neg	neg	
	P30			neg	$>2^{-10}$	$>2^{-10}$	$>2^{-10}$	2^{-5}	2^{-5}	2^{-1}	
9)	PGM	1B	2A1A	neg	1B2A1A	1B2A(1A)	1B2A(1A)	1B2A(1A)	1B2A(1A)		neg
	P30			neg	$>2^{-10}$	$>2^{-10}$	$>2^{-10}$	2^{-9}	$>2^{-10}$		2^{-9}
4)	PGM	1A	1A	1A	1A	1A		1A	neg	neg	
	P30			neg	$>2^{-10}$	$>2^{-10}$		$>2^{-10}$	$>2^{-10}$	2^{-5}	
10)	PGM	1A	1A	neg	1A	(1A)	1A	1A	(1A)	(1A)	neg
	P30			neg	$>2^{-10}$	$>2^{-10}$	$>2^{-10}$	$>2^{-10}$	2^{-8}	2^{-7}	2^{-6}
8)	PGM	1A2B	1B	neg	(1)	neg	neg	neg	neg	neg	
	P30			neg	$>2^{-10}$	2^{-8}	2^{-6}	2^{-3}	2^{-2}	2^{-1}	
5)	PGM	1A1B	2A1B	neg	neg	neg	neg	neg	neg	neg	
	P30			neg	2^{-10}	2^{-9}	2^{-9}	2^{-2}	1	2^{-1}	

P30 : last positive two-fold dilution of the extract
( ) : weak but still PGM subtypable band.

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Electrophoresis was run on cellogel, according to Sonneborn (1972) and stained according to Spencer et al. (1964).

Isoelectric focusing : polyacrylamide gels (5% T, 3% C, 220 x 110 x 0.5mm) were prepared with carrier ampholytes pH 4-6(LKB) 1 % (w/v) and pH 6-8 (LKB) 1 % (w/v). The anolyte and catholyte were 1M H_3PO_4 and 1M NaOH respectively. Samples were applied on Whatmann n° 1 filter papers, at 2 cm from the cathodal wick. Focusing was performed on the LKB Multiphor II, with Vmax: 1000 V, Imax: 10 mA, Pmax: 8.5 W, for 115 min. The gels were then PGM stained in the usual way. For some gels, the upper third was separately stained with Coomassie blue.

RESULTS

PGM Subtypes of Semen

72 samples of liquid semen were PGM subtyped and the observed phenotype frequencies were not significantly different from those found in the belgian population (Coosemans et al.,to be published). In 10 cases, the PGM subtypes of semen and blood were compared and no discrepancy observed.

Stability of Semen PGM in Experimental Stains

Semen stains of all PGM subgroups (except 2A2B) were made on cotton cloth with fresh semen and stored dried at room temperature. In all 72 recent stains the correct PGM subtype was determined. Out of 37 stains, analyzed after three months, 33 were PGM subtypable and 4 with initial lower PGM level in liquid semen were no more typable. After six months of storage, 11 stains out of 15 were still typable. After eight months, 5 stains out of 9 were still interpretable.

PGM Subtypes of Vaginal Secretions

23 semen-free vaginal swabs were analyzed. They were collected at least three days after sexual intercourse and were P30 negative. No blood was visible, although the benzidine test for blood was positive on some of them. Only 5 out of 23 samples had sufficient PGM activity to be subtyped. PGM activity was correlated neither with the delay before drying (immediate for 13 swabs, several hours for the others) nor with the delay before analysis (about four days for 10 swabs and about twenty five days for the others).

Postcoîtal vaginal swabs exhibited higher PGM activity since 5 out of 7 swabs collected within less than four hours after intercourse were PGM typable. Similarly 4 out of 5 swabs collected between 9 and 12 hours after intercourse were PGM typable, although semen PGM are known to be no more detectable after this delay. These swabs were dried after a few hours and typed after about 15 days.

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PGM SUBTYPING OF SEMEN AND VAGINAL SECRETIONS

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INTRODUCTION

The polymorphism of human phosphoglucomutase (PGM 1), well known in blood, has been shown to be present in semen and vaginal secretions (Culliford 1971; Brinkmann and Koops 1971). The extended polymorphism of PGM 1 later revealed in blood by isoelectric focusing, was reported in semen as well (Sutton 1979).

PGM activity in vaginal secretions is usually very low (Price et al. 1976; Eastwood 1977) but increases in the presence of semen (Garlo 1985). Other alterations of the PGM pattern in body fluid mixtures have been reported. The PGM group of semen is modified by saliva (Sensabaugh et al. 1980); this modification being reversed by addition of reducing agents.

In this work, the stability of PGM subtypes was studied in semen stains and postcoital vaginal samples, in view of their application to sexual assault evidence.

MATERIALS AND METHODS

Samples: Semen samples were obtained from the Urology Clinic, vaginal secretions samples from the Gynecology Clinic; coupled blood/semen/vaginal samples were collected from volunteers. Most liquid semen were frozen at -18°C within no more than 10 hours. Vaginal samples were collected with sterile cotton wool swabs. Semen stains and vaginal swabs were stored dried at room temperature. The presence of semen in the vaginal samples was assessed by a quantitative ELISA for P30 (Kamenev et al. 1987).

Extraction: For PGM, 50 mm2 of semen stain or of vaginal swab were extracted in 50 µl of a 0.1 % (v/v) 2-mercaptoethanol aqueous solution, for 1 hour at 20°C or alternatively overnight at 4°C. Liquid semen was diluted 1:3 with water containing 2-mercapto-ethanol at the same final concentration. This optimal concentration was previously assessed in semen samples of various PGM subtypes, mixed with saliva.

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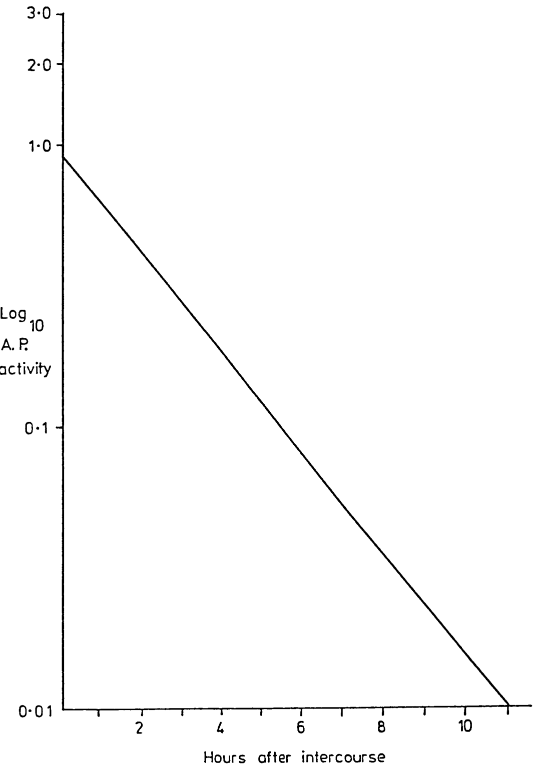

Fig. 6. Loss of acid phosphatase activity from semen after intercourse

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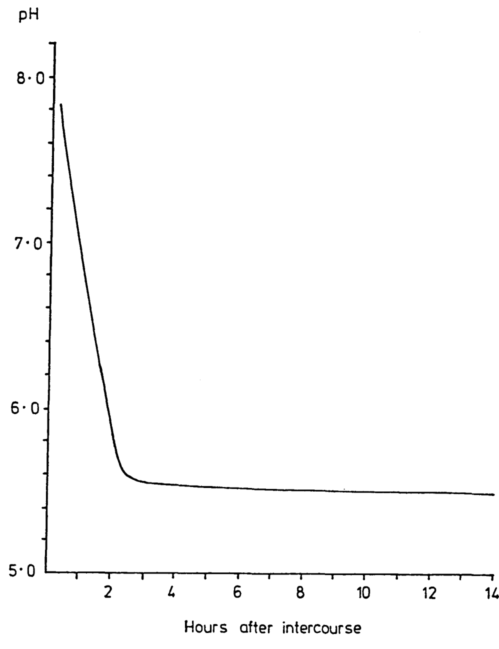

Fig. 4. Changes in vaginal pH after intercourse

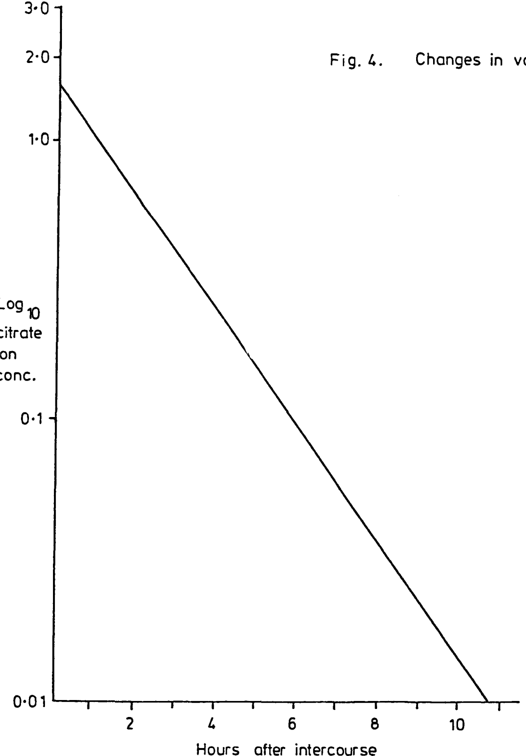

Fig. 5. Loss of citrate ions from semen after intercourse

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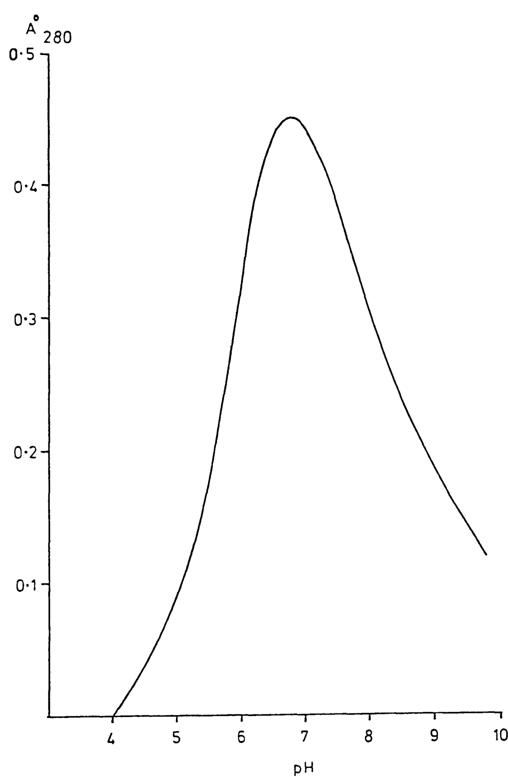

Fig. 2. Semen GLO activity in the range pH 4 0 to pH 9·5

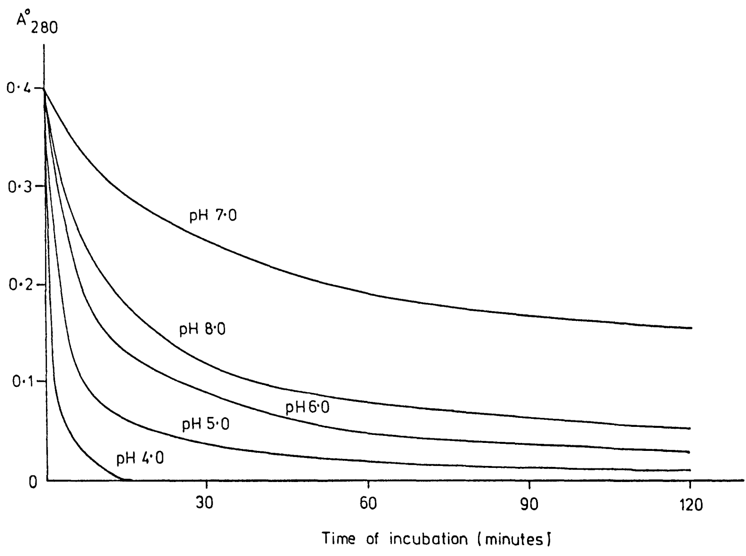

Fig. 3. The stability of semen GLO when incubated at pH 4·0 to 9·0 then readjusted to pH 7·0 for assay

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My conclusions are that a combination of drainage and loss of activity due to low pH are the main agents in producing loss of semen activity after intercourse.

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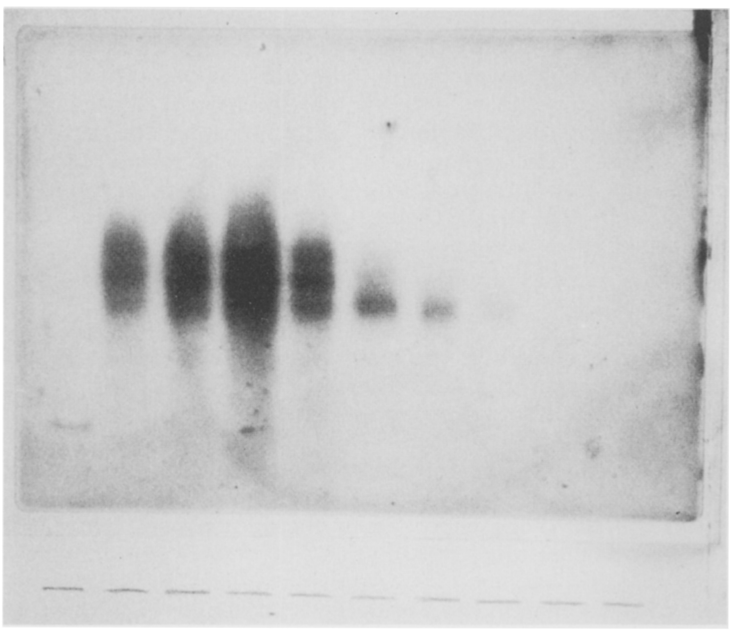

Fig. 1 GLO on vaginal swabs taken (left to right) - Pre-intercourse, immediately, ¼, ½, 1, 2, 3, 4, 6 and 7 hours post intercourse. (Female : GLO 1; Male GLO 2-1).

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yielding a major and two minor proteinase fractions. Each of these fractions had optimum pH activity at about pH 8.0.

These proteinase fractions were separately concentrated and incubated with semen aliquots at 37°C for 24 hours. No apparent loss of activity occurred when the semen was examined by electrophoresis.

(ii) pH studies

GLO was purified from pooled semen by DEAE sephacel and S-hexylglutathione affinity chromatography. The maximum activity of this GLO was found to be at about pH 6.5 to pH 7.0, with a fairly rapid decline of activity to pH 4.0 (Fig. 2).

The stability of semen GLO to pH shock was determined by incubation in the range pH 4.0 to 8.0 for 2 hours then, after adjusting the pH, assaying the GLO at pH 7.0. The results showed about 90% loss of activity after 2 hours incubation at pH 6.0 and about 97% loss at pH 5.0 (Fig. 3).

The pH changes of the semen pool in the vagina after intercourse were determined by taking serial vaginal swabs and measuring the pH at intervals up to 14 hours post intercourse. A rapid fall in pH occurs over the first 2 to 3 hours, when the pH falls from about pH 8.0 to about pH 5.5. After this initial period the decline in pH is much less rapid (Fig. 4).

(iii) Semen drainage

Davies and Wilson (1974) reported that the loss of seminal constituents from the vagina was mainly due to drainage, with the residual seminal components being diluted with vaginal secretions. Martin (personal communication) investigated the loss of acid phosphatase activity and citrate ions from post coital semen in the vagina. His results showed that both the citrate ion concentration and acid phosphatase activity fell by approximately 60% in the first 2 hours after intercourse (Fig. 5 and 6). This may be interpreted as being due to drainage.

Discussion

The rapid loss of semen GLO activity after intercourse severely restricts its use as a marker in cases of sexual assault. This loss of activity is unlikely to be due to the action of seminal or vaginal proteinases.

Concentrated neutral proteinases from the vagina did not produce any observed affect on semen GLO and acid proteinases demonstrated very little activity at the pH range recorded in the semen pool (pH 8.0 to pH 5.5) during the first 2 hours after intercourse.

The pH of the semen in the vagina falls to about pH 5.5 in the first 2 hours after intercourse and at this pH the activity of GLO falls by about 60%. Incubation at this pH followed by a readjustment to pH 7.0 for assay or electrophoresis, further reduces the activity due to pH shock. Over the time period up to 60% drainage of semen from the vagina has been noted.

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Post Coital Changes in Semen Glyoxalase I (GLO)

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Introduction

The frequencies of the commonly occuring GLO phenotypes are sufficiently high in all races to be used as blood markers in forensic science. (Emes and Parkin, 1980). Since GLO is also present in semen it has further use in the investigation of sex crimes.

Studies of human vaginal secretions showed that, apart from menstrual blood, GLO was absent from the unstimulated vagina.

The examination of post coital vaginal swabs from couples of different GLO phenotypes showed that the semen GLO activity was no longer detectable after a period of about 1 to 2 hours post intercourse, being replaced by GLO of the female phenotype (Fig. 1). This female GLO persisted for variable lengths of time up to about 10 hours post coitus, after which no GLO activity could be detected in the vagina.

The production of vaginal GLO appears to be due to sexual stimulation as it has been observed on vaginal swabs taken just before intercourse. Studies into the rapid loss of seminal GLO are described as this is not clearly understood.

Experimental

Three possible mechanisms have been investigated, (i) Breakdown by vaginal or seminal proteinases, (ii) inactivation by acid pH, (iii) semen drainage from the vagina.

(i) Vaginal and seminal proteinases

Acid and neutral proteinases have been described in semen and vaginal secretion (Lundquist 1952, 1955; Denker, 1977)

Acid proteinases:

The concentration of acid proteinases in semen were found to be about 15 times greater than in vaginal swab extracts. Acid proteinase was purified from pooled semen by the method of Ruenwongsa and Chulavatnatol (1975). The pH stability of this acid proteinase shows maximum activity at about pH 4.5 with a rapid loss of activity as the pH increases.

Neutral proteinases:

Neutral proteinases were partially purified from the pooled eluates from semen free vaginal swabs by sephadex G-200 column chromatography,

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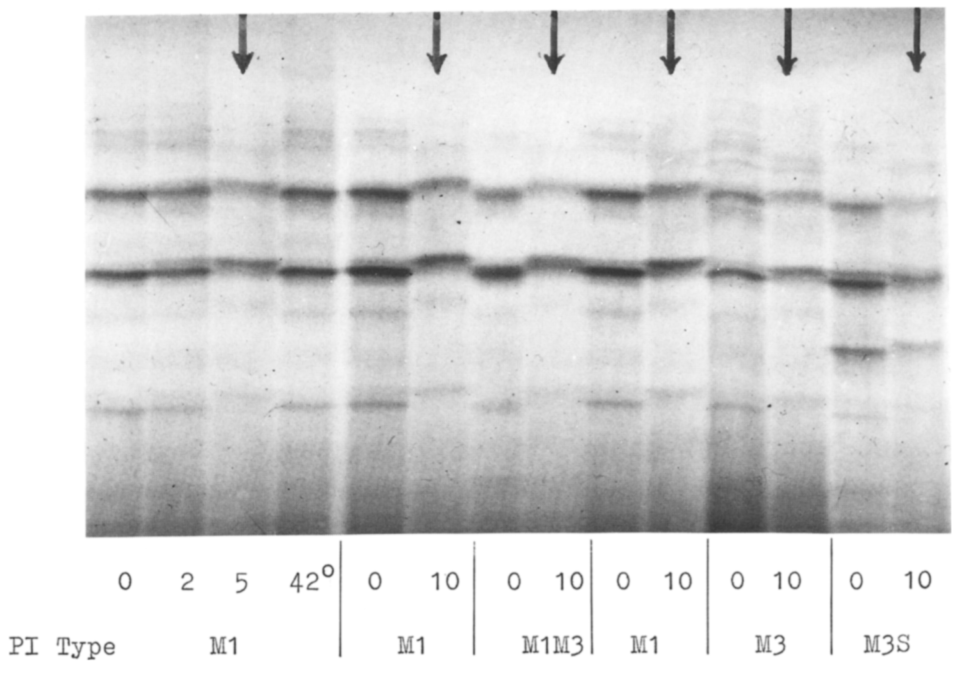

0 = Original
2,5,10 = UV Irradiation (min.)
42° = Heating (20 min.)
↓ = Conversion

Fig. 1. Effects of UV irradiation of serum samples on PI typing (IEF)

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Table 1. Effects of UV irradiation of serum samples on the typing of genetic polymorphisms

System	Method	Irradiation (min)	Effects on the specific protein bands
TF	AGE	1	distinctly reduced
		5, 10	strongly reduced or disappeared
C3	AGE	1	distinctly reduced
		5, 10	strongly reduced or disappeared
GC subtypes	IEF	1	unimportantly reduced
		5, 10	strongly reduced
TF subtypes	IEF	1	unimportantly reduced
		5	distinctly reduced
		10	strongly reduced
HP subtypes	IEF	10	distinctly reduced
BF subtypes	IEF	10	disappeared
PI subtypes	IEF	1	normal bands
		5, 10	all bands slightly shifted to the anode

Table 2. Effects of UV irradiation (10 min.) of serum samples on PI typing

Original PI type	IEF results after irradiation
M1	all bands slightly shifted to the anode
M1 M2	all bands slightly shifted to the anode
M1 M3	all bands slightly shifted to the anode
M3	all bands slightly shifted to the anode
M3 S	all bands slightly shifted to the anode
M3 M2	all bands slightly shifted to the anode → "new" phenotype like M1 M3
M2	all bands slightly shifted to the anode → "new" phenotype like M1

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For the exclusion of thermal effects we performed experiments in which serum samples were heated without using ultraviolet irradiation (42°C, max. 20 minutes). It was revealed that heating did not produce the effects of irradiation.

RESULTS AND DISCUSSION

The results are compiled in table 1. We could observe a reduction of the protein bands examined after ultraviolet irradiation using electrophoresis or isoelectric focusing as separation methods. This reduction may reach the degree of total disappearance of the protein zones (e.g. after 10 min. of irradiation). These results are like those obtained after different physical or chemical treatments for protein denaturation. These denaturations are due to changes of charge or structure of proteins.

Our results and the evidence obtained in this way for the different stability of proteins are in agreement with many other well-known facts in protein chemistry or in forensic serology.

The most important effects were found for the PI system. With all PI phenotypes tested, ultraviolet irradiation produced the same changes: all bands (zones 2, 4, 6, 7 and 8) were shifted to the anodic side (table 2, fig. 1). The distance of the shift corresponds to the distance between subtype M1 and M2 (corresponds to 0.01 pH units of the isoelectric point). We wish to underline this change because of the possibility that "new" phenotypes are produced by ultraviolet radiation (e.g. from PI M2 the "new" type "M1").
From this we conclude that there is a small change in the protein structure. Changes of glycolization have to be considered. We know that the different zones of PI after IEF are due to the different content in sialic acid (Yoshida and Wessels 1978).
But anodic PI bands contain a larger amount of sialic acid. For that reason the reported effects of irradiation cannot be explained by the loss in sialic acid.
For forensic serological practice we have to continue these investigations. The next steps will be the analysis of blood and of blood stains after ultraviolet irradiation.

SUMMARY

Short-term ultraviolet irradiation (5 or 10 min. with UVC) of serum samples is able to produce marked changes in the typing of genetic serum protein polymorphisms (C3, TF, GC, HP, BF, PI). For most protein bands varying degrees of reduction were observed which even went as far as their total disappearance. In the PI system small but important changes of the IP are obtained because "new" phenotypes arise from the original type (e.g. M1 instead of M2).

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Effects of ultraviolet irradiation on the electrophoretical typing of serumprotein polymorphisms

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INTRODUCTION

For many years a therapy has existed using the retransfusion of blood after ultraviolet irradiation (the socalled "ultraviolet autologous blood irradiation") (Wiesner 1967, Wiesner et al. 1986). The mechanisms of this therapy are still poorly understood. Up to now an extensive clinical study is still missing. Hence this therapy can rightly be regarded as a matter for scientific dispute.
The present study has the aim to investigate the effects after ultraviolet irradiation using a defined part of the human blood - the serum proteins. For that purpose standard methods for genetic typing were applied. These methods (e.g. isoelectrofocusing) are able to recognize very small differences in the structure or charge of proteins.
We hope to have thus made a contribution to understanding the effects of ultraviolet irradiation of the blood.
Moreover, a study of this type might provide information for the forensic serologist concerning the analysis of blood stains.

MATERIAL AND METHODS

Fresh serum samples were treated and analyzed. The treatment by ultraviolet irradiation was carried out in quartz cuvets with FMR 10 equipment (Manufacturer: VE Kombinat Präcitronik, Dresden, GDR, Hg-lamp HNU 6; maximum emission at 254 nm).
The periods of irradiation were 1, 5 and 10 minutes. The results of genetic typing were compared before and after irradiation.

The following serum protein polymorphisms were typed:

C3, TF	typing by agarosegel electrophoresis (AGE)
BF, GC, HP, PI and TF	including the subtypes typing by isoelectrofocusing (IEF) in polyacrylamide gel (0.5 mm) carried out with the "Multiphor" equipment (LKB, Bromma, Schweden). For technical details see Patzelt (1984), Prokop and Göhler (1986).

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ALPHA-1-ANTITRYPSIN
Gel size: 250x125x0.5 mm

Gel composition:
T=5% C=3%
glycerol 13% (w/v)
Pharmalyte pH4.2-4.9 6% (w/v)
ACES 0.05% (w/v)
Ammonium persulphate 0.05% (w/v)
TEMED 0.03%

Electrodic solutions:
Anolyte: 0.04 M Glutamic acid
Catholyte: 1 M β-Alanine

Bloodstains:
Each bloodstains(made with 10 µl of blood) was soaked overnight in 150 µl of 0.05 MDithiotreitol .Eluates were applied at 2 cm from the cathode by means of small papers (W 3MM 7x4 mm)

Running conditions:
2000 V, 16W, 20mA, 8°C
Focusing was carried out for 240 min(paper was removed after 60 min)

STAINING

Conditions reported by Carracedo et al.(1)were adopted for silver staining of Tf and Alpha-1-AT with the difference that we used shorter steps.

RESULTS AND DISCUSSION

Our results show that IEF and silver staining are reliable for determining Tf and Alpha-1-AT in forensic investigations on bloodstains.
With bloodstains keep at room temperature and frozen, the staining with Coomassie, for the above mentioned markers, given unreliable results: the bands were well detectable, but the phenotype was uniform according to the storage conditions.
By using silver staining, it was possible not only typing Tf and Alpha-1-AT two months old, keep at room temperature, but also, in minute bloodstains. Infact, we have obtained good results with stains diluted until 1:32. Frozen bloodstains are well typed for a few months.

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Tf and Alpha-1-AT typing in bloodstains by Isoelectric Focusing

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INTRODUCTION

Conventional staining procedure for serum protein have employed the use of Coomassie Brilliant Blue R-250, however, with this staining is impossible to detect these proteins when they are in small quantities, like in forensic samples, even if a highly sensitive method as IEF are used.

Silver staining, is recognized as a highly sensitive method for detect proteins in polyacrylamide gels. Infact, it was been demonstrated that for froensic purpose , silver staining is at least 100-fold more sensitive than Coomassie.

This report evaluated the use comining of PAGIF and silver staining as routine techniques for phenotyping Tf and Alpha-1-AT in bloodstains stored at various temperature.

MATERIAL AND METHODS

TRANSFERRIN:
Gel size: 250x125x0.2 mm

Gel composition:
T=5% C=3.2%
sucrose 12% (w/v)
Ampholine pH 5-7 3% (w/v)
Ammonium persulphate 0.05% (w/v)

Electrodic solutions:

Anolyte: 0.04 M Glutamic acid
Catholyte: 1M Ethanolamine

Bloodstains
Each bloodstains (made with 10 µl of blood) was soaked in 150 µl of 0.3% Ferrous ammonium sulphate solution for 3hours at room temperature. Eluates were applied by means small papers (W 3MM 7x4 mm) at 4 cm from the cathodal end.

Running conditions
1200 V , 20 mA, 3W, 6°C
Prefocusing 60 min
Focusing 150 min (papers were removed after 75 min)

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A SENSITIVE METHOD FOR SUBTYPING GC IN BLOODSTAINS.

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Isoelectric focusing in immobilized pH gradients was applied to the analysis of Gc variants in bloodstains. An immunoblotting technique with alkaline phosphatase conjugated antibody was used for the visualization of the Gc patterns. The sensitivity of the method was superior to that of methods employing immunofixation or protein staining, the detection limit being about 1 µg Gc protein/ml. The method was applied to typing of experimental bloodstains, and Gc phenotypes were detected in 8 months old stains. On testing 100 stains from casework only one discrepancy in repeated tests was detected.

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RESULTS AND DISCUSSION

The interpretation of the electrophoretic pattern for Hp 1, 2-1 and 2 phenotypes is possible in 5 µL of serum at a dilution of 1/512, 1/256 and 1/512 respectively (fig. 1).

The quantitative analysis was carried out by Immunochemistry System (nephelometric rate) and Mancini test. Table 1 shows the values obtained by these two methods. The electrophoretic determination is possible with serum containing 0.833 g/dL of Haptoglobin.

This micromethod is extremely sensitive compared to other electrophoretic techniques using the classical "pseudo-peroxidasic" stain (Hoste 1986). This author has detected the Hp phenotype 2-1 in 10 µL of serum at a dilution of 1/8. To obtain the same sensitivity limit achieved by our method, he has used the enzyme immuno-assay after blotting with three different antibodies.

In conclusion, this procedure is simple and sensitive and can determine Haptoglobin in bloodstains : we have found that 0.02 µL of blood was necessary with experimentally produced stains.

This technique is used as a routine method in our laboratory and has been proved to be successful in forensic case-work.

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Table 1. Values obtained by nephelometric rate and Mancini test for the three Hp phenotypes at increasing dilutions of serum.

	NEPHELOMETRY mg/dL			MANCINI mg/dL		
phenotypes :	1	2-1	2	1	2-1	2
serum dilutions						
1/2	31.4	41.3	13.5	91.5	106.0	57.3
1/4	16.6	23.5	6.43	50.5	63.5	35.0
1/8	8.4	11.1	3.47	-	-	-
1/16	4.26	5.28	1.68	-	-	-
1/32	2.24	2.9	0.86	-	-	-
1/64	1.16	1.36	*	-	-	-
1/128	*	*	*	-	-	-
1/256	*	*	*	-	-	-

*) $<$ 0.833 mg/dL   -) not detectable

Reference values : 27 - 139 mg/dL   70 - 380 mg/dL

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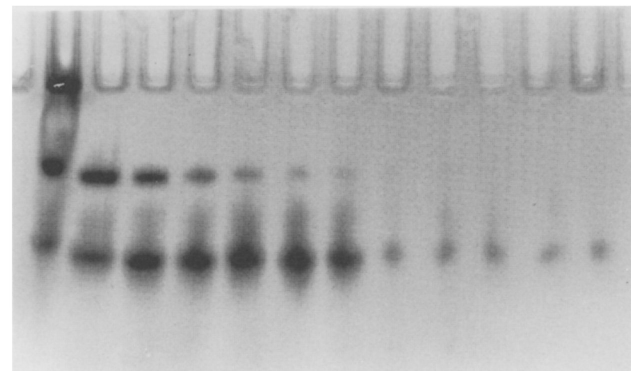
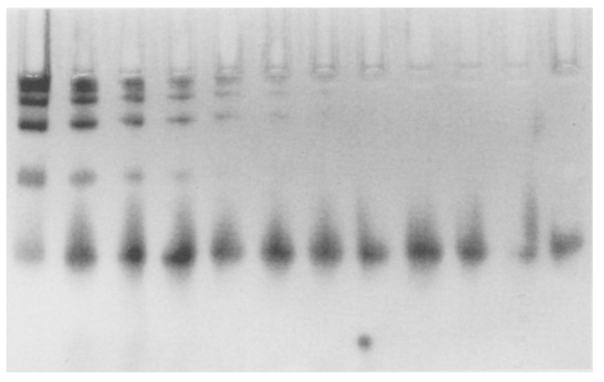


a)



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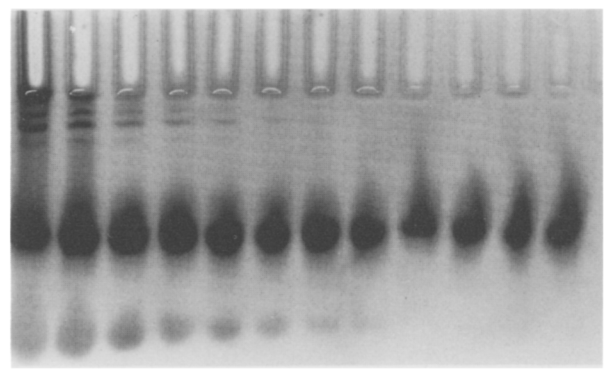

c)

Fig. 1 Electrophoretic pattern of Hp phenotypes at increasing dilutions of serum
a) Hp 1 b) Hp 2-1 c) Hp 2

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Sensitivity of a micromethod for Haptoglobin typing

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INTRODUCTION

The Haptoglobin phenotypes can be quickly and easily determined in bloodstains by an electrophoretic micromethod (Dimo et al. 1987), using a discontinuous buffer system according to Heredero (1974) with some modifications.
This new electrophoretic method presents some advantages : the gel preparation is simple and the precipitation of hemoglobin before electrophoresis is not necessary. Furthermore, this technique seems to be very sensitive. The determination of its sensitivity limit is presented here.

MATERIAL AND METHODS

Sample preparation

5 uL of sera samples of three Hp phenotypes at various concentration levels with hemoglobin traces were prepared.

Gel casting

The stacking gel (7.41% acrylamide, 0.19% bisacrylamide was immediately poured on the resolving gel (10.92% acrylamide, 0.28% bisacrylamide, 16.35% glycerol) in the Midget Twincast gel (LKB Instructions) prepared for two 0.75 mm thin gels with 15 well-former Combs.

Polymerization

One hour at room temperature

Electrophoresis

Discontinuous buffer system (Tris/Glycine) proposed by Heredero et al. (1974). Running at 40 mA constant current with two 0.75 mm thin gels.

Staining

O-Tolidine and hydrogen peroxide in acidic medium

Quantitative assay

Determination of the amount of Hp in three phenotypes at increasing dilutions of sera was carried out by rate nephelometric Immunochemistry System (Beckman Instrument) according to Sternberg (1977) and by radial Immunodiffusion (Mancini et al. 1975)

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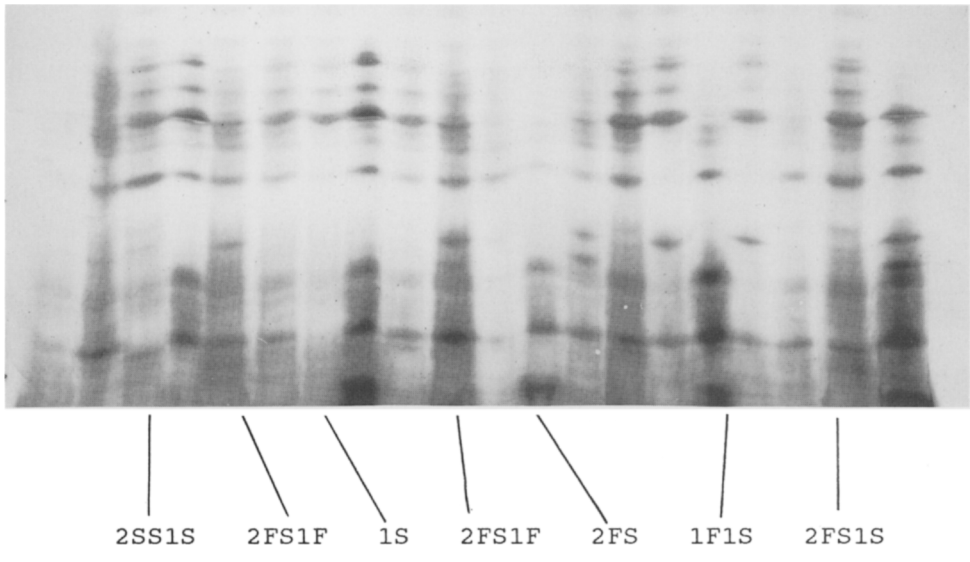

Figure 2. Haptoglobin subtyping of 8 months old control bloodstains.

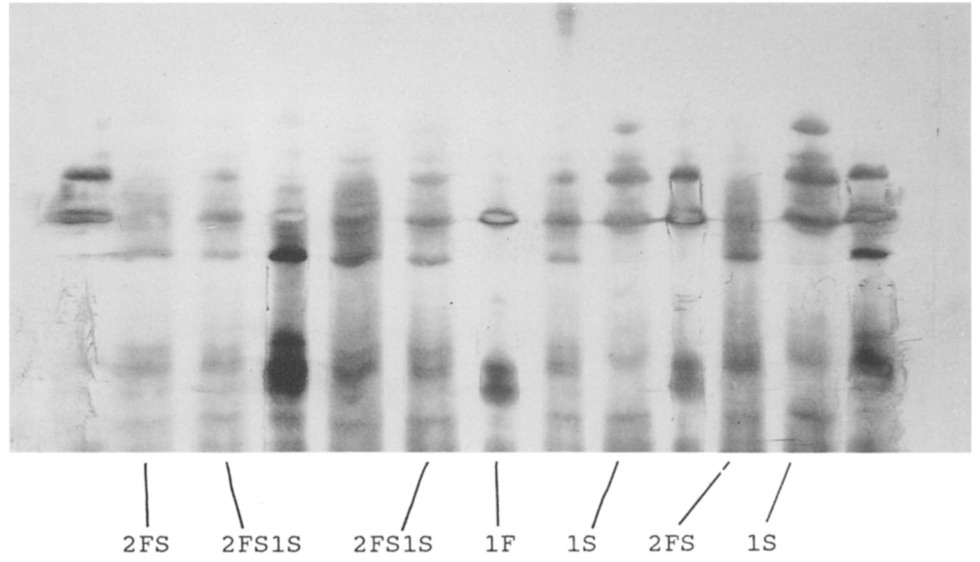

Figure 3. Haptoglobin subtyping of bloodstains from case-material.

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with the results of the ordinary haptoglobin typing using a polyacrylamide gradient gel. No discrepancies were observed. The subtyping of control bloodstains and bloodstains from casematerial gave immunoblots showing additional bands and more background staining, compared to the results of serum-samples (figures 2 and 3). The backgroundstaining sometimes masked haptoglobin bands of weak samples; treatment of the samples with solutions containing different amounts of neuraminidase in order to reduce the backgroundstaining gave no improvement. Sometimes the additional bands caused difficulties interpreting the results. In some instances ordinary haptoglobin typing was necessary to come to a conclusion. However in casematerial the quantity of bloodstain is limited. Therefore supplementary ordinary haptoglobin typing cannot always be done. In those cases it will not always be possible to give a reliable result of the haptoglobin subtyping. Further investigations will be necessary to overcome the described problems and then haptoglobin subtyping using isoelectric focusing can be a valuable tool in forensic bloodstain investigation.

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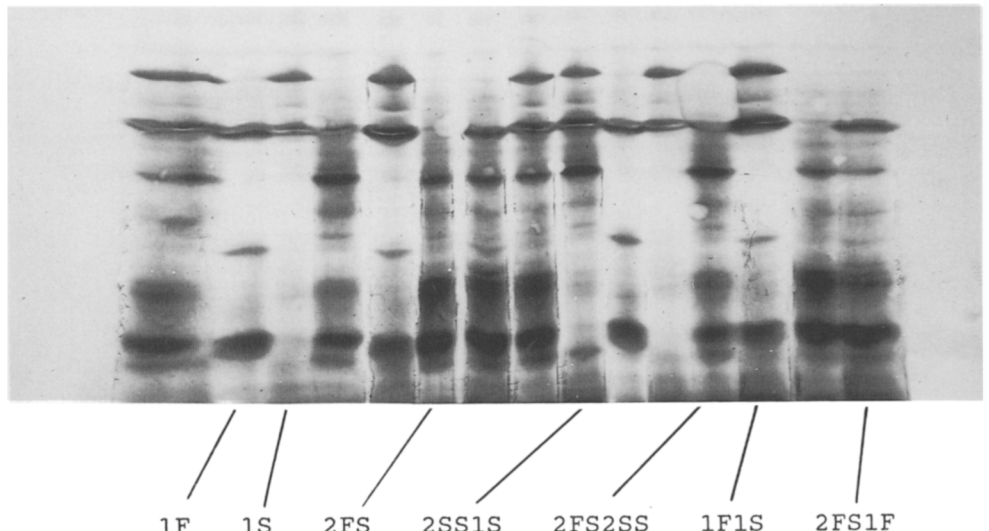

Figure 1. Haptoglobin subtyping in sera.

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Electrophoresis

Gelpreparation

Polyacrylamidegels, 120 x 260 x 0.5 mm, were cast on the hydrophobic side of a GelBond PAG-film (LKB). Our gel contained 5.8 ml acrylamide/bisacrylamidesolution (T5C3), 8.4 ml aquadest, 40 µl TEMED (Merck), 580 µl persulphatesolution (100 mg/ml), 275 µl ampholine pH 3.5 - 5 (LKB), 275 µl ampholine pH 5 - 7 (LKB) and 100 µl ampholine pH 3.5 - 10 (LKB).

Isoelectric focusing

Isoelectric focusing was performed at 6° C. The electrode distance was 10 cm and the electrode solutions were 1M NaOH for the cathode and 1M H_3PO_4 for the anode. The samples were applied on sample application paper (Pharmacia), 3 x 10 mm for the stain samples and 5 x 2 mm for the serum samples, 1 cm from the cathode. Prefocusing was carried out for 1 hour (settings 500 V, 8 W). Focusing with the samples was carried out for 1 hour (settings 1500 V, 8 W) and without samples for 1½ hour (settings 2000 V, 8 W).

Blotting procedure

A nitrocellulose membrane filter (Schleicher & Schuell, West Germany) was presoaked in aquadest and put on top of the gel. (The nitrocellulose will adhere firmly to the gel.) The membrane and the peeled off gel are then subjected to electroblotting, using a Trans Blot Cell (BioRad) at 70 V for two hours. As blotbuffer was used: 6.06 g TRIS, 28.8 g glycine, in 400 ml methanol and 1600 ml aquadest. After blotting the nitrocellulose membrane was washed in a PBS-Tweenbuffer (13.6 g Na_2HPO_4 $2H_2O$, 4.9 g KH_2PO_4, 17.2 g NaCl and 3 ml Tween (Merck) in 2 l aquadest, pH 7.2) for 2 hours. The membrane is put in a 1:500 PBS-Tweenbuffer dilution of anti human haptoglobin, IgG fraction goat (Atlantic antibodies) to shake overnight. The next day the membrane was washed in several changes of PBS-Tweenbuffer for one hour before being subjected to a second antibody solution (125 µl peroxidase conjugated rabbit anti goat IgG (1g/ml, Behring) in 25 ml PBS-Tweenbuffer).
For the visualisation of the Hp-band the membrane is put in a solution of 30 mg 4-chloro-1-naphtol in 3 ml acetone, mixed with 50 ml of a 0.2 M NaCl, 59 mM TRIS-HCl buffer, pH 7.4 and 40 µl 30% H_2O_2 is added.

RESULTS AND DISCUSSION

Serumsamples from about 500 unrelated persons were investigated according to the described method. A result of a subtyping is shown in figure 1. The immunoblot shows a good distinction between the different subtypes, although it is sometimes difficult to distinguish between a 2FS and a 2FS-2SS subtype. The results of the subtyping by isoelectric focusing were compared

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HAPTOGLOBIN SUBTYPING IN BLOODSTAINS

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INTRODUCTION

Haptoglobin typing is routinely performed in numerous forensic laboratories for bloodstain investigation. Recently methods have been published of haptoglobin subtyping in fresh human serum using isoelectric focusing (1, 2). Compared to the conventional method, the discriminating power of haptoglobin subtyping using isoelectric focusing raises from 0.62 to 0.76 and therefore the new method becomes very promising in forensic bloodstain investigation. In this study we investigated the possibility of haptoglobin subtyping of bloodstains on a polyacrylamide gel by isoelectric focusing, using electroblotting and immunoperoxidisase visualization of the haptoglobin bands. Control bloodstains, stored at roomtemperature for up to eight months, and the validity of the system in casework were investigated.

MATERIALS AND METHODS

Preparation of the samples

Bloodstains

Cotton cloth was stained with blood from individuals with known haptoglobin types. The stains were stored at roomtemperature for up to eight months. Bloodstains from casework were kept at -20° C until investigation. A small piece of bloodstain (appr. 5 x 5 mm) was wetted with 15 µl neuraminidase (from Clostridium perfringens, Boehringer). After one hour at roomtemperature the wetted bloodstain was centrifuged. The extract was left overnight at 36° C. The next day 40 µl of 8 M urea solution containing 2% v/v ampholine pH 3.5 - 10 (LKB) and 10% v/v mercaptoethanol was added to the bloodstain extract. The samples were then frozen at -70° C for about two hours before applying on the gel.

Sera

5 µl serum was mixed with 5 µl of the neuraminidase solution. The procedure was then as described above.

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present being tested for stains. In practice, the different extraction conditions make the use of microtitre plates uneconomical and microtubes must be used for maximum efficiency.

Until the name DNA fingerprinting (Jeffries 1985) has been justified with respect to bloodstains and is equally as sensitive as these systems described here, it is felt that this approach offers an extremely reliable and flexible alternative.

	Phen	%	Nondisc.
Gc	1S	34	0·259
Tf	C1	57	0·406
PLG	1	51	0·388
α_2HS	1	43	0·395
FXIIIA	1	62	0·416
FXIIIB	1	54	0·408
Pi	M1	55	0·368
Hp	2FS	34	0·228
		0·25	0·0002

DI=0·9998

fig. 1. List of protein systems used, the frequency of the commonest phenotypes, and the individual and combined discrimination values.

IMMUNOCHEMOPHORESIS

PRETREATMENT
↓
IEF
↓
ELECTROBLOT
↓
ANTISERUM
↓
ENZYME-LINKED-ANTIBODY

fig. 2. Outline of the immunochemophoresis technique.

Sensitivity

	Vol.(µl)	max. titr	Stain(m)
Gc	2	800	18
Tf	5	64	6
PLG	5	5	6
α_2HS	5	*	6
FXIIIA	2	16	*
FXIIIB	2	32	6
Pi	5	8	6
Hp	5	*	*

* still under investigation

fig. 3. Comparison of the sensitivity of each system listing the volume of sample applied, the maximum dilution obtained and the age of blood stains (months).

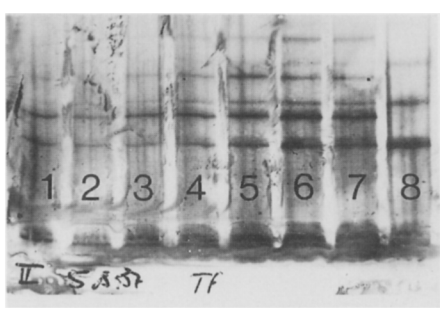

fig. 4. Titration series for Transferrin type C1-B. Sample 7 is undiluted serum followed by sequential doubling dilutions to sample 1, a 1/64 dilution. Sample 8 is TfC3-1.

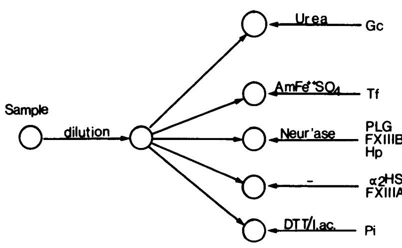

fig. 5. Scheme of extraction and pretreatment of samples using a micro-method.

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Extensive testing and comparative studies (Rand 1987) have revealed the considerable advantages of this method in - contrast to passive blotting methods for example.

An outline of the method is shown diagrammatically in fig. 2, and is preceded by an appropriate pretreatment or extraction process for stains depending on the protein tested.

Visualisation of the protein bands is carried out using a specific antibody, an enzyme-linked antibody-complex and substrate as outlined by Pflug (1986). The method is principally the same for all the systems except that the type of pretreatment, the optimal pH range, electrophoretic conditions and the antiserum dilutions are varied for optimal results within each system. A detailed description of the method for Gc (Rand 1987) has already been published. Methods for the other systems are in progress.

The maximum sensitivity of each system was investigated by doubling dilution series and the results are listed in fig. 3. A typical dilution series is shown in fig. 4 for Tf. The sensitivity of detection depends not only on the amount of protein present in blood but more important on the quality i.e. the avidity and affinity of the antisera.

As has been well documented the detection of a protein polymorphism in serum or plasma does not necessarily predispose its detection in blood stains. This depends mainly on the stability of the protein in the dried state or the ability to reconstitute or reverse any changes which have occured during this process.

The systems described can all be detected in stains and after appropriate pretreatment, or extraction, no appreciable differences to serum samples have yet been detected.

Studies are still being carried out, but Gc has been detected in laboratory-made stains in 1 1/2 year-old stains and FXIIIB, Tf and Pi in 6 month-old stains. Testing of the various systems under varying storage conditions (4°, 37°, humid chamber etc.) are still under examination but has as yet produced only a weakening of bands without the generation of anomalies.

DISCUSSION

The application of semi-dry electroblotting and immunochemical visualisation of proteins has increased the sensitivity and reliability of detection of many polymorphic protein systems so that they can now be considered for use in blood stain grouping. From the optimal dilution factors seen in fig. 3 it can be estimated that it would be possible to detect these systems in approximately 2 µl of serum. This in round figures would mean approximately 5-10 µl of blood stain. Quantitative experiments on stains are to be carried out to assess this value but it seems to be relatively realistic.

A micro-extraction procedure using micro-titre plates, as outlined in figure 5, has been tested for serum samples and is at

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An approach to individualisation of micro-bloodstains using immunochemophoresis

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INTRODUCTION

One important limiting factor for the individualisation of bloodstains is the size. When the size of the bloodstain is in the micro-litre range the choice of blood group systems to be tested becomes critical and for the traditional systems such as ABO, Gm, PGM etc. the size of the stain dictates which, and how many of these systems can be used. The choice depends, among other things, on the sensitivity of the system.

The application of polymorphic DNA-markers or "fingerprinting" would seem to be excluded from the investigation of these micro-stains because of the present requirements of stain examination. Gill (1985) has estimated 60 µl of blood are necessary and Baird (1986) approximately 100 µl although this figure will obviously be reduced with the advent of new detection methods.

By the application of immunochemophoresis to the detection of polymorphic protein systems such as Gc, a substantial increase in the sensitivity can be achieved and the inclusion of these systems in the repertoire for bloodstain grouping would be advantageous for individualisation.

The 8 polymorphic protein systems described here (fig. 1) have a combined discrimination value of 0,0002 or 99,98 % (1 in 5.000) and the most common phenotype combination occurs in 0,25 % of caucasians.

These proteins have all been extensively studied in serum samples but up till now only Gc is in general use for stain grouping. The other systems are at present undergoing the necessary stringent examination before acceptance can be possible.

METHOD

The term immunochemophoresis has been adopted to describe the combination of electrophoretic (IEF) separation and immunochemical detection. The latter necessitates the transfer of proteins to an inert carrier and in this case the transfer is carried out by semi-dry electro-blotting onto a nitro-cellulose membrane.

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Table 3. Detectability of GM allotypes and recovery of serum albumin from dried bloodstains

	PERCENT RECOVERY OF ALBUMIN							
	OUTSIDE				INSIDE			
Stain Contaminant	1	2	3	4 -weeks-	1	2	3	4
NONE	24	15	8	8	-	97	45	33
GASOLINE	14	13	6	8	-	80	47	37
GREASE	42	43	9	11	-	95	51	40
NEW OIL	30	54	30	19	-	95	51	40
USED OIL	ND	38	6	11	-	91	49	28
BRAKE FLUID	4	10	10	12	-	89	50	34
SEMEN	6	8	5	6	-	87	50	41
URINE	3	6	4	6	-	52	27	7

00 = Complete GM phenotype detected (underlined)
00 = Partial phenotype/ no allotypes detected

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Table 1. Detectability of GM allotypes in dried bloodstains[1] contaminated by various substances and stored indoors at ambient temperature.

Contaminant	Allotypes absent at week: 1	2	3	4	6
None	N[2]	N	N	N	N
Gasoline	N	N	N	N	N
Grease	N	N	N	N	N
Clean Oil	N	N	N	N	N
Used Oil	N	N	N	N	N
Brake fluid	N	N	N	N	N
Dirt	N	N	N	N	N
Semen	N	N	N	N	N
Urine	N	N	N	A.B0	A,X,F.B0,G,G5

[1] Bloodstains prepared with blood of phenotype GM: A,X,F.B0,G,G5
[2] N = None absent

Table 2. Detectability of GM allotypes in dried bloodstains[1] contaminated by various substances and stored outdoors.

Contaminant	Allotypes present at week: 1	2	3	4	6
None	A,X,F.B0,G,G5	A,X,F.G.G5	X,F	X	N[2]
Gasoline	A,X,F.B0.G.G5	A,X,F	N	N	N
Grease	A,X,F.BO.G.G5	A,X,F.G.G5	N	N	N
Clean oil	A,X,F.B0,G,G5	A,X,F.G	A,X,F.G	A,X,F.G	X,F
Used oil	A,X,F.G5	A,X,F.G5	N	N	N
Brake fluid	X.G5	N	A,X,F.B0,G5	X,F	N
Dirt	A,X,F.B0.G,G5	ND[3]	A,X,F.B0,G5	X,F	N
Semen	X.G5	N	G5	N	N
Urine	G5	N	N	N	N

[1] Bloodstains prepared with blood of phenotype GM: A,X,F.B0,G,G5
[2] N = None detectable
[3] ND = Not done

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temperature for the duration of the study period. The other stain panel was covered with clear plastic wrap and placed on the roof of the laboratory building facing due south. At weekly intervals, replicate bloodstains were removed from each test and control area of the indoor and outdoor arrays for determination of the GM allotypes present. Stains were extracted for one hour at room temperature in one ml of PBS containing 1% bovine serum albumin. The concentration of albumin in the bloodstain extracts was determined by enzyme-linked immunosorbent assay [unpublished observations].

RESULTS AND DISCUSSION

The detectability of allotypes in blood deposited on blue jeans that were maintained in the laboratory for the duration of the study period (Table 1), was affected only by the presence of urine. By the fourth week after preparation, two thirds of the allotypes were undetectable in bloodstains mixed with urine; by the sixth week, none could be detected.

A quite different picture of allotype detectability was seen with the identical array of bloodstains that had been maintained outdoors (Table 2). After only one week, incomplete phenotypes were obtained for blood that had been mixed with used motor oil, brake fluid, semen, and urine. The ensuing weeks of storage rendered the allotypes in other bloodstain/contaminant mixtures undetectable. By the sixth week of study, the only allotypes detectable were X and F in blood contaminated by clean motor oil.

In a companion series of studies, it was noted that the recoverability of albumin from extracts of these bloodstains appeared generally to diminish in parallel with the inability to detect the allotypes (Table 3). More albumin was recoverable from the bloodstains kept indoors, and these stains also yielded the greatest number of extracts with complete GM phenotypes.

These studies indicate that some substances that can commonly contaminate evidentiary materials are not likely to affect the detection of the GM allotypes as long as the evidence has been maintained under reasonable conditions of temperature and humidity. The outdoor exposure of bloodstains to direct sunlight for as little as one week in the presence of several contaminants (e.g. used oil, semen or urine) can be expected to result in a loss of allotype detectability.

The inability to extract albumin from the outdoor stains suggested to us that the allotypes might not have been destroyed by the adverse conditions, but had become undetectable because of resistance of the bloodstain immunoglobulins to extraction. This belief was supported by the observation that detectability of some of the allotypes could be restored 30 weeks after outdoor storage if the extractions were carried out for 14 days (data not shown).

The present study corroborates the observations of others, that the GM allotypes present in dried bloodstains are robust. GM allotypes were readily demonstrable in dried stains kept at room temperature for at least six weeks despite contamination by adventitious substances. Within this category of stains, only urine appeared to have a deleterious influence on allotype detectability. Although extremes of temperature and humidity, in concert with some contaminants, appeared to compromise the detectability of GM allotypes in dried stains, much of this behavior could be attributed to a probable inability to readily solubilize the immunoglobulins rather than actual loss of antigenicity.

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PERSISTENCE OF GM ALLOTYPES IN BLOODSTAINS EXPOSED TO ADVERSE CONDITIONS

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INTRODUCTION

The inherited structural differences in the heavy chains of IgG, known as GM allotypes, can be exploited serologically for the partial genetic characterization of bloodstain evidence [Ducos et al 1963; Khalap et al 1976; Kipps 1979]. The favorable distribution of phenotypes in United States populations [Schanfield 1987], and the long term stability of these antigens in dried bloodstains [Hoste et al 1978] were factors that encouraged an evaluation of the GM allotyping methodology for potential use in the FBI Laboratory.

This report is a portion of that evaluation and describes the results of our assessment of the detectability of GM allotypes in bloodstains contaminated by various substances and stored under conditions potentially deleterious to proteins.

MATERIALS AND METHODS

Allotyping procedure

Bloodstain extracts were tested for GM A,X,F.B0,C3C5,G,G5 by the method of Schanfield [1987]. All tests were performed in V-bottom microplates by the sequential addition of 25 uL bloodstain extract, 25 uL anti-allotype antiserum, and 25 uL anti-D coated group O R_1R_1 erythrocytes. The plates were incubated at 4^0C for one hour followed by centrifugation at 1000 RCF for one minute. The test results were read macroscopically 10-20 minutes after tilting the plates at an angle of 60^0. Positive and negative control samples were run simultaneously on each microplate. All allotyping reagents were obtained from Allotype Genetic Testing, Atlanta, GA.

Bloodstain preparation and storage

Clean, well-worn denim blue jeans served as the fabric substrate for the preparation of bloodstains. Defined areas on both legs of the jeans were treated with each of the following substances: gasoline, lubricating grease, brake fluid, unused motor oil, used motor oil and ground-in red clay (dirt). Within two hours of the application of these substances, multiple 25 uL aliquots of fresh whole blood (GM phenotype: A,X,F.B0,G,G5) were pipetted onto each of the contaminated areas of the jeans. In other areas, blood deposition was followed immediately by the addition to the stains of either semen or urine. Bloodstains were placed also in in uncontaminated regions of the fabric. The stains were allowed to dry overnight at room temperature. The following morning, the jeans were split to obtain duplicate stain panels. One panel was maintained in the laboratory at room

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Table 1

G1m(f) Results
Serum/Stain Samples

	HAI	NIB	
	Serum	Serum	Stain
Positive	90	91	86
Negative	113	113	116
Inconclusive	5	4	6

Table 2

G1m(f) Results
Stored Stains

	Positive	Negative	Inconclusive
App. 18 mo.			
NIB	30	27	3
Serum sample by HAI	32	26	2
2-10 years			
NIB	7	5	4
Serum sample by HAI	11	4	1

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conjugates evaluated include alkaline phosphatase (ALP) and horseradish peroxidae (HRP). ALP substrates, unlike HRP, showed little reactivity with endrogenous enzymes found in serum/plasma.

Table one lists the results on the serum/stain samples. Inconclusive results by HAI include three samples that had a positive antibody screen and two in which duplicate testing did not agree. The NIB inconclusive results include four serum samples which were HAI Glm(f) negative, and five stain samples which were HAI Glm(f) positive.

Table two lists the results on the stored stain samples.

CONCLUSION

The NIB procedure we describe was found to be suitable for both serum/plasma samples and bloodstains. Advantages of testing for Glm(f) by NIB include the following: 1) low set up cost, 2) permanent record of results, 3) greater sensitivity and specificity, 4) minimal technician time/ skills, and 5) no interference with antibodies.

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Evaluations made in developing the NIB procedure included membrane type, protein block, dilution of antibodies, and enzyme conjugate.

The NIB procedure used for this study was as follows:

1) Apply 2.5ul sample on nitrocellulose, dry overnight.
2) Block 1 hour in TBS (pH 7.5), 3% Liquid Hipere Gelatin, 5% Tween-20, .1% Hammersten casein.
3) Wash membrane 3 X 5 minutes in wash buffer (1% block solution in TBS).
4) Add to membrane 1:6000 dilution monoclonal anti-Glm(f) (BAM 17 from Seward Laboratory, England) in wash buffer. Agitate gently for 1 hour at room temperature.
5) Wash membrane 3 X 5 minutes in wash buffer.
6) To membrane add 1:12000 dilution of alk phos conjugated goat anti-mouse IgG (Sigma) in wash buffer. Agitate gently for 1 hour at room temperature.
7) Wash membrane 3 X 5 minutes in TBS.
8) Wash membrane X1 in stain buffer (TBS, pH 9.1).
9) To membrane add substrate mixture of NBT/BCIP in ratio 1:3 for 30 minutes; NBT (75mg NBT, .7ml dimthylformamide, .3 ml H_2O) and BCIP (50mg, 1 ml dimethylformamide).
10) Wash membrane briefly in deionized water blot dry.
11) Dry completely before scoring.

Bloodstains were extracted by placing two 4mm threads in 75 ul of 3% BSA for 1 hour at room temperature. Dilutions of 1:2, 1:5, and 1:10 were prepared.

Serum/plasma samples were prepared by making dilutions of 1:100, 1:200, and 1:300 in saline.

Positive and negative controls were run on each membrane in the following dilutions: Serum - 1:100, 1:200, 1:400, 1:800, 1:1000, 1:2000; Stains - Neat, 1:2, 1:5, 1:10, 1:20, 1:40. These controls were assigned a value based on the color development assessed visually on a scale of 0 to 4.

Each of the three dilutions on the unknown tested was assigned a numeric value by two readers after comparison to the controls. The values determined by each reader were added. The results were interpreted as follows: 0-5 - negative; 6-14 inconclusive/ repeat; and 15-24 - positive.

RESULTS

Membranes evaluated for use in the NIB procedure included: Biodyne (nylon), Genatran - 45 (nylon), Zetaprobe (nylon), Immulon (polyvinyl), and S & S nitrocellulose. The nitrocellulose membrane had the clearest background, the least non-specific binding, and was the least expensive. The enzyme

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G1m(f) Immunoblot Procedure

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Immunoglobulin allotyping can be valuable for discrimination purposes in the forensic laboratory. Current Gm typing procedures include hemagglutination-inhibition (HAI) and enzyme linked immunosorbant assay (ELISA). HAI, the most commonly employed method, has some disadvantages including a high frequency of interferring antibodies as well as being a labor intensive test. The ELISA test method, in microtiter plates, eliminates some of the problems with HAI. A drawback of this system is its technical complexity. To simplify the ELISA technique we have developed a nitrocellulose immunoblot (NIB) procedure.

MATERIALS AND METHODS

Bloodstains were prepared on clean cotton cloth from 208 blood samples (126 Blacks, 30 Caucasian, 31 Amerindian, 21 Mexican-American) drawn for the purpose of parentage testing. Serum/plasma was separated and stored at 4°C. All samples were tested for G1m(f) by HAI during routine paternity testing.

Sixty bloodstains stored at room temperature for 18 months were used as well as 16 bloodstains stored from 2-10 years. The blood samples from which these bloodstains were prepared had been tested for G1m(f) by HAI during routine paternity testing.

For routine paternity testing HAI is performed in V-microtiter plates as follows:

1) 25ul of appropriately diluted antiserum is added to test wells, 25ul of 3% BSA in saline is added to screen wells.
2) 25ul of a 1:20 dilution of serum is added to test wells and screen wells.
3) Plates are incubated at 18°C for 30 minutes.
4) 25ul of 0.2% coated red cells are added to all wells.
5) Plates are incubated at 18°C for 30 minutes.
6) Plates are centrifuged.
7) Plates are set on a 60° slant board for 5-10 minutes.
8) Plates are read by streaming.

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TABLE 7

FORENSIC STAIN ANALYSIS

Semi-quantitative IgG estimation and Ig allotyping

Case

Victim : V

Accused : A

Pieces of evidence : bloodstain x
bloodstain y

Titer in HAI

	z	a	x	f	n	g	b°	Km1	Km3	IgG	IgG (ug/ml)
Serum V	+	+	-	+	+	+	+	-	+		
Serum A	-	-	-	+	+	-	+	+	+		
Control bloodstain V	4	4½	0	3	5½	2	2	0	>6	6½	28
Control bloodstain A	0	0	0	3½	>6	0	3	2½	>6	7	37
Stain x	4	4	0	3½	5½	3	3	0	>6	7	37
Stain y	1	0	0	1	3	0	1	0	4	4	4

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TABLE 6

DETECTABILITY OF Gm AND Km ANTIGENS IN RELATION TO IgG CONCENTRATION IN LIMITED DILUTED BLOODSTAIN EXTRACTS

Minimal IgG concentration required for the detection of allotypes present in limited diluted stain extracts.

allotype	phenotype	minimal IgG concentration		
		number tested	range ug/ml	mean ug/ml
z	zaxg	1		9,2
z	zafngb	18	3,4 - 12,0	7,6
a	zaxg	1		9,2
a	zafngb	18	3,6 - 31	14,0
x	zaxg	1		4,6
x	zaxf(n)gb	18	2,2 - 18,6	7,4
f	fnb	35	4,6 - 18,6	11,6
f	zafngb	18	9,2 - 31	17
n	fnb	35	3,4 - 26,4	11,4
n	zafngb	18	4,6 - 18,4	9,4
g	zaxg	1		13,2
g	zafngb	18	9,2 - 19,6	14
b^0	fnb	35	2,4 - 18,4	7,0
b^0	zafngb	18	3,2 - 9,8	6,6
Km(1)	Km1+3+	12	6,6 - 37,2	23,4
Km(3)	Km1-3+	65		<0,6
Km(3)	Km1+3+	12		<0,6

The minimal IgG concentration required for the detection of Gm and Km antigens in diluted extracts of control bloodstains is obtained from the IgG concentration in the extract and the maximum dilution at which the allotype present in the extract could be detected.

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TABLE 1. REAGENTS FOR Gm AND Km TYPING

anti-allotype	manufacturer	batchnr.	anti-D-batchnr.
anti z	CLB	3272	3471
anti a	CLB	3294	3471
anti x	CLB	2984	3545
anti f	CLB	2871	3480
anti n	CLB	R120	n-protein 1)
anti g	CLB	4040	3285
anti b^{o}	CLB	3955	2127
anti Km(1)	CLB	3951	2447
anti Km(3)	CLB	3674	3597

1) The IgG2 (n+) myeloma protein (code Jas) was a gift from Dr. G. de Lange from the CLB.

TABLE 2. SEROLOGICAL PROPERTIES OF REPRESENTATIVE ALLOTYPING REAGENTS

Anti-Allotype Antiserum				O R_2R_2 Sensitization	
allotype	antiserum	titer(3)	working dilution(4)	reagent	optimal concentration
z	anti z	160	60	anti D (za)	1:1
a	anti a	160	40	anti D (za)	1:1
x	anti x	80	20	anti D (x)	3:1
f	anti f	160	60	anti D (f)	1:1
n	anti n	1600	400	n-protein (2)	0,08 mg/ml
g	anti g	160	60	anti D (g)	3:1
b^{o}	anti b^{o}	160	40	anti D (b)	1:1
Km(1)	anti Km(1)	140	80	anti Km(1)	3:1
Km(3)	anti Km(3)	160	60	anti Km(3)	1:1

1. The optimal anti-D concentration is the optimal sensitizing volumeratio of anti-D to saline using a cell concentration of 10% group O R_2R_2 cells in the reaction mixture.
2. The optimal sensitizing amount of n-protein is the concentration of n-protein in saline with 10% group O cells and 0,02% $CrCl_3$ in the reaction mixture.
3. The titer of the anti-allotype antisera is expressed as reciprocal dilution giving the last 1+ agglutination reaction with optimally sensitized cells.
4. The optimum anti-allotype antiserum dilution (working dilution) was obtained from a two dimensional titration scheme with the anti-allotype antiserum titrated against dilutions of Gm (+) and Gm (-) control sera.

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given in table 7. The table summarizes the results of the allotypic marker testing conducted on reference blood samples from victim and accused. The evidence consisted of two blood stains (stain x and y).

DISCUSSION

This study was undertaken by our laboratory in order to get the availability of a reliable and feasible immunoglobulin allotyping method. Allotyping was performed by a haemagglutination-inhibition test. For forensic purposes the test must be as sensitive as possible. The inhibition method can be optimized by using optimally diluted antisera. Firstly we selected appropriate allotyping reagents. The working dilutions of anti-allotype antisera and corresponding anti-D coats were obtained. The features and serological properties of a representative set of allotyping reagents are given in table 1 and 2. Optimally diluted allotyping reagents were firstly extensively tested with sera and bloodstain extracts both negative and positive for the relevant factor before the reagents were released for staintyping in actual casework. The limits of sensitivity in terms of the amount of bloodstain extract which is needed for the positive identification of alloantigens was thoroughly investigated. Our approach to this problem was the measurement of the concentration of IgG, the protein on which the allotypes are actually located. IgG was measured by an agglutination-inhibition method. This method was sufficiently sensitive and accurate for our purposes. The quantification of IgG appeared highly useful in bloodstain allotyping. Knowing the actual IgG concentration in the extract, negative inhibition results of the extract in the inhibition test for allotyping could be interpret by using the figures of table 6 where the detectability of Gm and Km antigens in relation to the IgG concentration in the bloodstain extract are given. The relative performance of the alloantigens in the HAI test was also used for the interpretation of negative reactions. The presence of a certain positive marker can rule out any possibility of an immunoglobulin-subclass deficiency. A representative example of an actual case is given in table 7. The IgG concentration in the stainextract from stain x allows positive identificattion of all allotypes when present. From this it was substracted that the phenotype of the blood on stain x was Gm (fnb), Km (1-, 3+), the same type as the blood from the victim.
The low IgG concentration in the bloodstain extract of stain y did not allow to report the true absence of the factors Gm (a, x and g) and Km(1).

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Agglutination-inhibition method for semiquantitative IgG estimation

Doubling dilutions of stain extract were made in microtiter plates. To 25 ul of stain extract dilution 25 ul optimally diluted IgG antiserum (usually 1 : 300 in saline) and 0,1% Coombs control cells in 5% FCS were added successively. Incubation and reading of the plates were analogous to the procedures for determination of allotypes.

RESULTS

Serological properties of allotyping reagents

The optimal sensitizing anti-D dilutions along with the titers of the anti-allotype antisera were determined in one operation using two-dimensional titration schemes. Subsequently, the optimum anti-allotype antisera dilutions (working dilutions) were obtained from two-dimensional titration schemes with the anti-allotype antisera titrated against dilutions of control-sera both positive and negative for the relevant factor using optimally coated red cells. The optimum dilutions of the allotyping reagents which are listed in table 1 are given in table 2. The addition of Foetal Calf Serum to the agglutination-reaction prevented adherence of red cells to the plates. In many different batches of FCS unspecific reactions due to its addition were never encountered.

Detectability of Gm and Km antigens in relation to IgG concentration in limited diluted bloodstain extracts

To determine the sensitivity of Ig allotyping in bloodstain analysis a large series of extracts of control bloodstains with several different Gm/Km antigen compositions were tested. Used bloodstains (n = 77) were not older than 6 weeks in this experiment. The heat treatment of the bloodstain extract effectively eliminated occasionally occurring unspecific agglutinations of the extract with the coated red cells only, but had no effect on the titer of the extract in het HAI reactions for IgG estimation or Ig allotyping. Limiting dilution experiments were conducted to determine the maximal dilution of the bloodstain extract at which the respective allo-types present in the bloodstain could be detected. From this the minimal IgG concentration required for the detection of the respective alloantigens present in the bloodstain extract was established (table 6). The relative performance of the different alloantigens can also be substracted from this table. For example: Less than 0,6 ug/ml IgG in the stain extract is required for Km(3) detection while at least fortyfold more IgG in the extract is required for Km(1) detection. The amount of IgG which is required for the detection of the respective Gm antigens lies between 4,6 and 17 ug/ml IgG. As the sensitivity of the HAI test can be affected by the quality and the freshness of the coated red blood cells, it is essential to determine the sensitivity of the test for any alloantigen in each new experiment.

Ig allotyping of bloodstains in casework

Tests for Ig allotyping were made on a large number and variety of actual case material in conjunction with semiquantitative IgG estimation. In most circumstances it was possible to interpret negative inhibition results of the bloodstain extract by this results of the IgG determination. A representative example is

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INTRODUCTION

In forensic medicine the determination of immunoglobulin allotypes is valuable for the identification of bloodstains. A recurrent problem in Gm typing is the interpretation when no inhibition of the anti-allotype antiserum occurs (Khalap, 1979 and Schmitter, 1980). This result can represent the true absence of the Gm factor or the concentration of the marker in the extract was too low for detection. In this paper we report the application of a sensitive semi-quantitative IgG estimation in relation to the detectability of the Gm factors (z, a, x, f, n, g and b^0) and the Km factors (1 and 3). The relative performance of the individual antigens in serum and stain extracts is also studied.

MATERIALS

Anti-allotype antisera, their corresponding anti D-coats and controlsera both positive and negative for the relevant allotype were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB) in Amsterdam. A representative set of allotyping reagents is given in table 1. Foetal Calf serum (heat inactivated) was from Flow Laboratories. V-bottom microtiter plates were purchased from Greiner. Coombs Control cells and anti-human IgG (H-chain specific) were from Ortho Diagnostics Systems. Standardserum (HOO-03) for the quantitative determination of serumproteins was obtained from the CLB.

METHODS

Extraction for bloodstain typing

0,5 cm^2 Of bloodstained fabric was extracted in 1,5 ml of saline at roomtemperature for 1 hour. The mixture was heat treated at 65°C for 10 minutes, frozen at -20° C, thawed and the supernatent was centrifugated until clear.

Agglutination-inhibition method for determination of allotypes

Allotyping of sera was done in at least three dilutions of the serum (1 :15, 1 : 45 and 1 : 135). Serum dilutions were heat treated for 10 minutes at 65°C, frozen at -20°C and centrifugated until clear. Samples of stain extracts were usually tested in doubling dilutions starting undiluted. In microtiterplates 1 drop (25 ul) of serum dilution or stain extract, 1 drop of optimally diluted antiserum and 1 drop of allotype-coated 0,1% red cells in 5% FCS were added successively. Plates were mixed and left overnight at 4°C. Agglutination reactions were read macroscopically with the help of a light box after being placed for 10 minutes at an angle of 60°. The highest dilution of stain extract giving complete inhibition of agglutination is read. Controls included always: saline controls to show sufficiently strong agglutination of the coated red cells with the anti-allotype antiserum, positive serum controls, negative serum controls and a control for the serum or extract with the coated red cells only, which must not show agglutination.

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APPLICATION OF IMMUNOGLOBULIN ALLOTYPING IN FORENSIC STAIN ANALYSIS; RELIABILITY AND SENSITIVITY OF Gm AND Km TYPING.

"The demonstration of the factors Gm (z, a, x, f, n, g and b^{o}) and the factors Km (1 and 3) in relation to the Immunoglobulin G concentration in the stain extract".

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Abstract

This paper reports the detection of a wide scope of immunoglobulin alloantigens in bloodstains. Commercially available antisera and their corresponding anti-D reagents were evaluated in agglutination-inhibition tests for bloodstain grouping in forensic serology. The determination of a maximum of different allotypes increased the value of immuno-globulin allotyping in forensic bloodstain analysis. Particular reference is made to the application of a sensitive semi-quantitative IgG estimation in relation to the detectability of the respective allotypes. The relative performance of the individual antigens in serum and extracts of fresh and aged bloodstains were obtained. On the basis of these results it was often possible to interpret negative reactions of the bloodstain extract in the hemagglutination-inhibition method with confidence. There is no evidence for the preferential loss of any of the alloantigens after drying and after aging of the blood.

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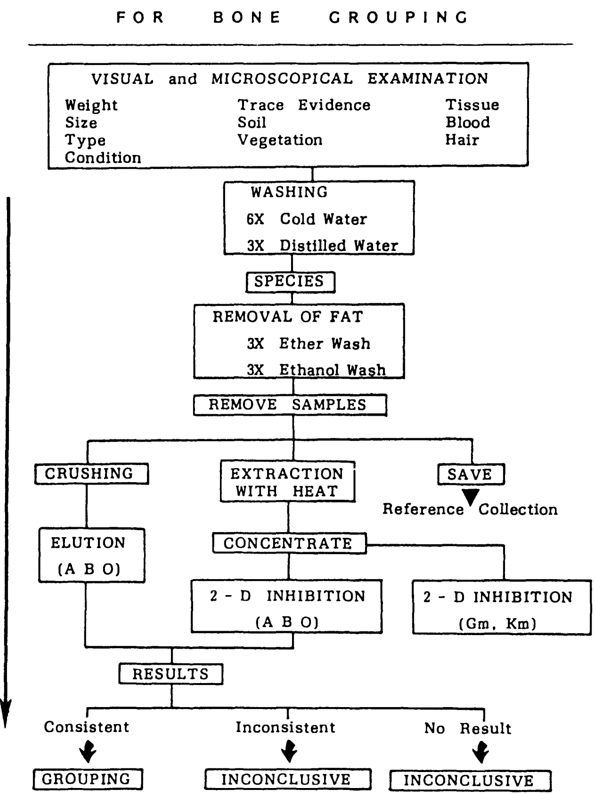

Fig. 3. Overall Procedure for Bone Grouping and Interpretation of the Results

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A

SMALL FRAGMENTS

Air Dry until Ether - Free

10 - 15 mg Sample

DIRECT ELUTION

B

Extract Small Fragments in Boiling Distilled Water for 1 - 2 hours

FILTER THE EXTRACT

REMOVAL OF FAT

EVAPORATE EXTRACT TO AN APPROPRIATE CONCENTRATION

2 - DIMENSIONAL ABSORPTION - INHIBITION

OR

INDIRECT ELUTION

Fig. 1. Preparation of Bone Samples for Grouping Procedures

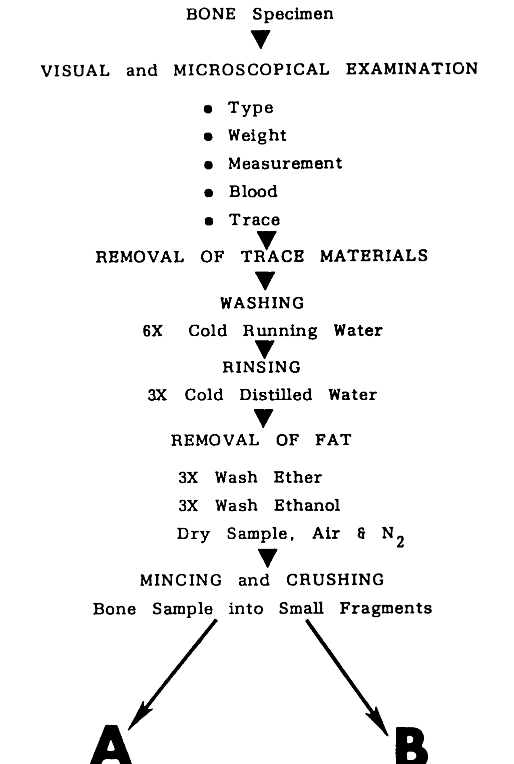

Fig. 2. Preparation of Bone Samples for Direct Grouping or Extraction for Indirect Grouping

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Table 2. Absorption Elution Results on Bones from Persons of Known ABO Blood Group

Type of Bone	ABO Type	n	Result[a] A	B	H	AB	NAD	INC
Fresh	A	19	19	-	-	-	-	-
	B	19	-	18	1	-	-	-
	O	33	5	-	27	-	-	1
	AB	-	-	-	-	-	-	-
Older	A	10	4	-	-	1	5	-
	B	10	-	5	-	1	4	-
	O	19	-	3	10	-	6	-
	AB	2	-	-	-	1	1	-

[a] NAD = no antigen detected; INC = inconclusive

Our results to date are consistent with those of previous investigators in indicating that neither elution nor inhibition (including the two dimensional) procedures alone yield uniformly correct typing results from bone samples.

Figure 3 shows a representation of our current overall approach to bone grouping as developed to the present time. If a blood group is assigned only in cases where the sample yields conclusive results with both elution and inhibition, and where the results are consistent with each other, no erroneous typing results have been observed in our studies thus far.

Studies on bone grouping are continuing in our laboratories to test additional samples for ABO antigens, and to determine whether the Gm/Km antigens can be determined in bone tissues. Inhibition procedures as well as ELISA techniques can be used for Gm/Km antigen typing. In addition, DNA can be isolated from fresh human bone samples, and its nature and quantity in this tissue are also being studied.

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METHODS

Bone samples for analysis are first examined visually and microscopically for possible blood or other trace materials, and the type, size and weight of the sample are recorded. Samples are then washed successively in tap and distilled water, and in several organic solvents to remove fatty tissues. The sample is then minced or crushed into small fragments, which may be subjected to direct elution testing, or to further preparation for extractive inhibition testing (Figs. 1 and 2).

RESULTS and DISCUSSION

Results of testing 21 fresh and 37 older bone samples from persons of known ABO type by extractive two dimensional absorption inhibition are shown in Table 1. "Older" bone samples are those six months old or older.

Table 1. Absorption Inhibition Results on Bones from Persons of Known ABO Blood Group

Type of Bone	ABO Type	n	Result[a] A	B	H	AB	NAD	INC
Fresh	A	6	6	-	-	-	-	-
	B	4	-	4	-	-	-	-
	O	11	-	-	11	-	-	-
	AB	-	-	-	-	-	-	-
Older	A	10	9	-	-	-	1	-
	B	10	-	9	-	1	-	-
	O	15	-	-	12	-	3	-
	AB	2	-	-	-	2	-	-

[a] NAD = no antigen detected; INC = inconclusive

Of 58 samples tested, 54 yielded conclusive results. No antigens were detected in the remaining four. All fresh bones and all except one older bone giving conclusive results were correctly typed. One sample from a group B person yielded an A + B antigen result.

Results of testing 71 fresh and 41 older bone samples from persons of known ABO blood group are shown in Table 2. Of 71 fresh bone samples tested, all but one yielded conclusive results. However, one group B sample yielded H antigen, and 5 group O samples yielded A antigen results. With 41 older bone samples, 25 yielded conclusive results and no antigen was detected in 16 others. Five of the 25 samples giving conclusive results, however, gave incorrect results.

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The Typing of ABH Antigens in Human Bone

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INTRODUCTION

Forensic serologists are occasionally called upon to perform ABH grouping tests on human bones or bone fragments, usually to aid in the identification of human remains. The antigenic receptors which characterize the ABO system occur not only on the surfaces of blood cells but are widely distributed throughout human tissues (Schiff 1931; Hartmann 1941). Over the years, a number of reports on ABH grouping of various tissues have appeared (Gaensslen 1983; Slavik and Meluzin 1970; Troger et al. 1976) primarily using procedures which were devised originally for grouping ABH antigens in bloodstains. However, the application of blood grouping techniques to bone tissue has yielded mixed results, particularly when aged bones or bones subjected to putrefactive processes have been analyzed. Earlier investigators utilized absorption-inhibition procedures (Boyd and Boyd 1934; Candela 1936; Thieme and Otten 1956, 1957), while absorption-elution techniques have been utilized almost exclusively in more recent attempts to type ABH antigens in bone tissue (Borgonini 1968; Yada et al. 1972; Beyer 1982; Berg et al. 1983; Hauser et al. 1984). The percentage of incorrect results in these studies has been so high, however, that the procedures could not be regarded as reliable. Greater but still limited success has recently been reported by Hauser (1986) using a semiquantitative elution technique which we (Gaensslen and Lee 1984; Gaensslen et al. 1985a, 1985b) and others (Lincoln 1973; Lincoln and Dodd, 1973) have previously described and recommended for the evaluation of antisera for bloodstain grouping and for the grouping of blood group antigens other than ABH in bloodstains. Recently, we have explored a combination procedure for ABH typing of bone involving extractive absorption-inhibition and direct absorption-elution procedures (Lee et al. 1987). Because of the sensitivity required, we have found that the only extractive inhibition component of any value in this overall procedure is our recently described two-dimensional method (Lee et al. 1986; and see R.E. Gaensslen & H.C. Lee in these Proceedings).

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The freezing process:
2 ml-s of samples are cooled to +4 C^{O} and then frozen at about 1 C^{O}/1 min.

Stored: at -40 C^{O}

Thawing: at room temperature /without any shaking or warming/.

Washing the thawed cells /in four steps, using equal volume of samples and washing solution/.

1st step washing solution:	12 ml of glicerine 88 ml of 5 % sodiumcitrate
2nd step washing solution:	5 ml of glicerine 35 ml of 5 % sodiumcitrate
3d step washing solution:	1:1 volume of the solutions of 1st and 2nd step
4th step washing solution:	physiological sodiumchloride

Attention:
- to prevent the haemolysis, the washing solution and the pellet have to be in the same volume;
- in the examinations which need serum containing medium for the red blood cells, after washing;
- the cells are separated by centrifugation /2000 rpm, 5 minutes/;
- the physiological salt solution is carefully discarded;
- the red blood cell pellet is resuspended in neutral AB serum or in 5 % of albumin solution to have the prefered concentration of cells.

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The Application of the Frozen Erythrocytes in the Bloodstain Diagnostic

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A more exact, easier and safer laboratory process is possible using frozen red blood cells for the determination of the eluated antibodies in the routine work of forensic serology laboratories.

A greater quantity of red blood cells with known antigen properties can be saved frozen for longer time.

The cells are from the Blood Transfusion Service where all of the donors are tested for AIDS and Hepatitis B, so the risk of the laboratory infections can be lowered.

The activity and specificity of frozen cells is the same as for the fresh cells.

Requirements for the indicator red blood cells which are to detect the eluated antibodies in routine forensic serological laboratories:

- known antigenicity /antigen-properties/
- donors have to be screened for AIDS and Hepatitis B /to protect the laboratory personnels/.

Easy to work with a serie of red blood cells, in which different samples contain different antigens, and the same "standard" can be used for a longer time.

For this reason a quantity of red blood cells with known antigen properties is distributed to small samples and saved frozen for future tests.

In such a way collected and frozen test cell panel can be used to detect the eluated antibodies of: /against/

ABO, Rh/Cc, D, Ee/, M, N, S, s, Le^a, Le^b, Fy^a, Fy^b systems.

The freezing of the red blood cells:
ACD or CPD stabilised cells are mixed with the freezing solution in 1:1 volume.

The freezing solution:

Sodium citrate x $2H_2O$	30,0 g
Sodium dihidrogenphosphate x $2H_2O$	3,1 g
Disodiumhidrogenphosphate x $2H_2O$	2,8 g
solved in aqua destillata /destillated water/	600,0 ml
add glicerine	400,0 ml

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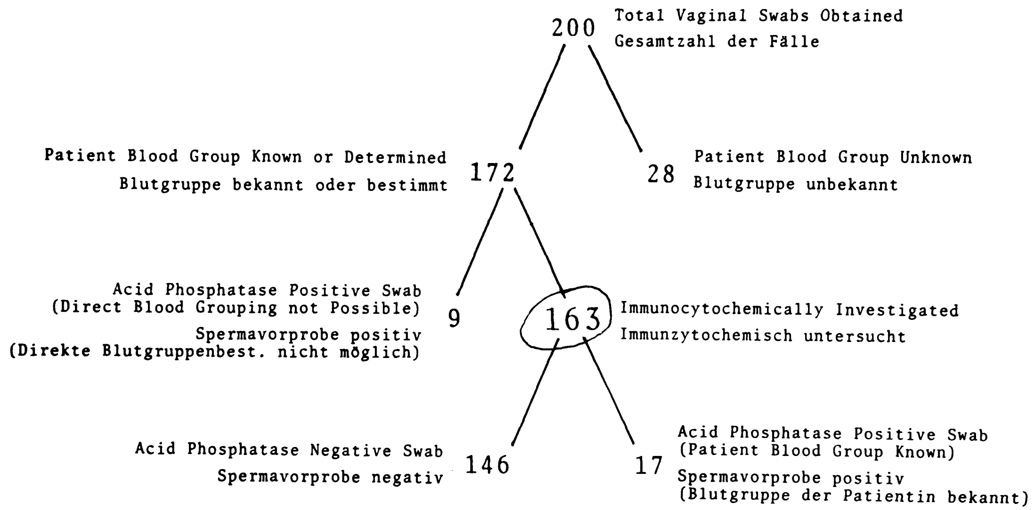

Table 1:

Investigated Cases / Untersuchte Fälle

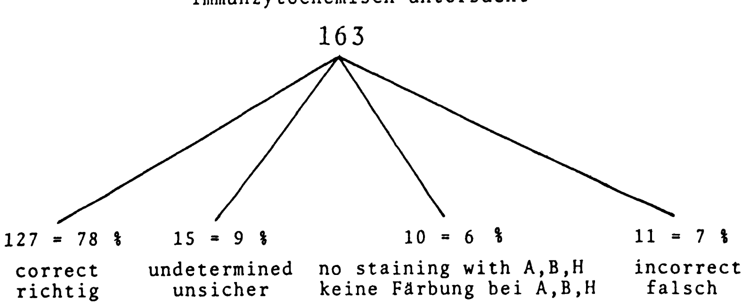

Table 2:

Summary of Results / Gesamtergebnis

	n*	correct richtig	undetermined unsicher	no reaction keine Färbung	incorrect falsch
Secretors Ausscheider	122	100 ≙ 82%	10 ≙ 8%	3 ≙ 3%	9 ≙ 7%
Nonsecretors Nichtaus-scheider	27	16 ≙ 59%	4 ≙ 15 %	6 ≙ 22%	1 ≙ 4%

* Only negative acid phosphatase vaginal swabs used
Nur Fälle mit negativer Spermavorprobe

Table 3:

Results Dependent on Secretor Status
Abhängigkeit der Ergebnisse von der Ausscheidereigenschaft

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RESULTS AND DISCUSSION

Each case was only stained once, and read by a person not knowing the expected result. Table 2 shows a summary of these results. In 78 % of the cases, the correct blood group was determined from the immunocytochemically stained vaginal cells. In 9 % of the cases, a blood group determination was not possible, because there was an insufficient amount of difference between the cell reactions and background staining. Perhaps this rate could be reduced with improved technique. In 6 % of the cases, there was no staining with anti-A-, anti-B-, or anti-H-serum. In 7 % of the investigated cases, an incorrect blood group was obtained, for unknown reasons.

The best results were obtained from patients between 21 and 40 years and from pregnant patients. In general, more mature vaginal cells with a relatively smaller size nucleus, are better for blood group determinations, rather than the more immature cells with larger nuclei.

Because the swabs investigated were obtained from gynaecology clinic patients, the vaginal cells of many cases showed a higher degree of cytolysis, which obviously diminishes the quality of immunostaining.

The last and probably most interesting aspect to be discussed, is the dependence of the results on secretor status. Table 3 shows the large number of cases of non-secretors which did not react. The investigation of vaginal swabs alone cannot explain this phenomenon. The results of a not yet published study (Scheithauer and Spiegelsberger) will illustrate the reason: All the cell layers of vaginal epithelium of secretors are completely marked, with respect of blood group. However, in non-secretors, only the intermediate and parabasal cell layers are labeled. Therefore, in order to correctly determine ABH blood group in non-secretors, vaginal swabs must contain cells from deeper levels of the mucosa. Hence, it's obviously impossible to determine secretor status from a vaginal swab by ABH staining alone.

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Immunocytochemical ABH Blood Group Staining in Vaginal Swabs

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INTRODUCTION

In blood grouping of mixed secretions from two or more persons, attributing results to the individual is always a problem. During investigations of sexual assaults, it would be greatly advantageous to be able to determine the blood group of single vaginal epithelial cells, for example discovered on the penis of a suspect.
Although the mixed cell agglutination reaction technique (Cooms et al. 1956) was a possibility, immunostaining (Sternberger et al. 1970) seems to be the method of choice for this purpose. Brinkmann et al. (1986) published an initial report about ABH blood group typing of single cells, with encouraging results.

MATERIAL AND METHOD

In the present investigation, vaginal swabs from 200 gynaecology clinic patients were used. Necessary blood group information could be obtained directly from the patients (n = 57) or through direct (n = 27) or indirect testing (n = 116). 28 of these 116 cases, obviously non-secretors, could not be classified and were not stained. Secretor status was indirectly determined on all swabs tested. Only acid phosphatase negative (sperm negative) swabs were used, or acid phosphatase (Berg 1957) positive swabs, if blood group information of the woman was known (Table 1).
Each vaginal swab was spotted onto three separate areas of a glass microscope slide, and incubated in parallel with anti-A-, anti-B-, and anti-H-sera. Immunostaining was performed by a four step technique ((monoclonal anti-A-, anti-B-, anti-H-serum (anti-A and anti-B from Biotest, anti-H from Fresenius), anti-mouse-serum from the rabbit (Bionetics), anti-rabbit-serum from the swine (Dakopatts), peroxidase-anti-peroxidase complex from the rabbit (Dakopatts), AEC as substrate)).

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The two sorts of anti-A sera used in the later work did not distinguish between A type 1 and A type 2 synthetic antigens and it is commonly thought that most anti-A and anti-B sera react with their relevant determinants on both type 1 and type 2 chains. In contrast, the structure of the H antigenic determinant is profoundly affected by the type of linkage between the β-D-galactose and the β-N-acetyl-D-glucosamine residues (Lemieux 1982) and it is thought that the Ulex europaeus anti-H lectin, commonly used for grouping body fluids, only reacts with H type 2, not H type 1 (Pereira et al 1978, Sugh et al 1982, Lemieux 1982, Le Pendu et al 1982).

If this is so and if the secreted blood group substances in semen are much the same as those in saliva, then this could be the reason for the relatively poor grouping reactions of O secretor seminal stains. As Ulex europaeus anti-H lectin is used in this laboratory, only a portion of the H antigenic determinants in semen would be detected. Grouping of A, B and AB secretor semen may be more successful because the anti-A and anti-B sera react with both type 1 and type 2 antigenic determinants. If vaginal fluid is more like serum than saliva, then the observation of low levels of H blood group substance in the sera of OLe^b individuals could be an explanation for the extremely poor grouping reactions of O secretor vaginal material, especially as the anti-H used only detects a portion of the small amount present.

IMPLICATIONS

The implications of the findings regarding secretor semen and vaginal material are as follows.

1. When interpreting the ABH reactions of seminal stains, due weight must be given to the possible contribution of any vaginal material present. The strength of the contribution will depend not only on the amount of vaginal material and the secretor status of the female, but also on the ABO group.

2. As grouping of A, B and AB secretor semen is usually successful particularly if the stain is strong, in some circumstances absence of reaction may be interpreted as absence of a particular group of semen.

3. As grouping of weak stains of A, B and AB secretor semen is fairly successful, failure to group such stains may result in lost evidence.

4. Although grouping of O secretor semen is far less successful than that of A, B and AB secretor semen, nevertheless there appears to be far more detectable H substance in O secretor semen than in vaginal material of the same group. Potentially this means that the strength of the H reaction might be a way of identifying O secretor semen when mixed with O secretor vaginal material.

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Strong and Weak Seminal Stains

The stains in the survey were classed as strong, moderate or weak according to the strength of the acid phosphatase (AP) reaction and the numbers of spermatozoa present on a slide made from the stain extract. Strong stains were defined as those with a high level of AP activity and abundant spermatozoa and weak stains as those with little or no AP activity and few spermatozoa.

Table 2 - The numbers of conclusive reactions obtained from strong stains of secretor semen.

A secretor	B secretor	AB secretor	O secretor
34/40	38/41	11/11	19/34

A + B + AB secretor = 83/92 = 90%
O secretor = 19/34 = 56%

It was found that most strong stains of A, B and AB secretor semen gave conclusive reactions, together with just over half of the strong stains of O secretor semen (Table 2). A large proportion of the failures were vaginal swabs. (It was obvious in this survey, as it had been in previous work (Davies 1982) that vaginal swabs were less useful for determining the seminal ABO group than were stains on other items.)

Just over a third of weak stains of A, B and AB secretor semen gave conclusive reactions (37%, 26/71), whereas only one weak stain of O secretor semen gave conclusive H reactions (1/17), and that was a penile swab where other secretions could have contributed to the reaction.

SECRETOR VAGINAL MATERIAL

Table 3 - The number of conclusive reactions for the group of the vaginal material, obtained from mixtures of it and semen.

A secretor	B secretor	O secretor
70% (144/207)	49% (42/86)	8% (6/76)

Grouping of A secretor vaginal material was at least as successful as that of semen of the same group (Table 3). It seemed that B secretor vaginal material gave conclusive B reactions rather less often and the O secretor vaginal material gave conclusive reactions only occasionally.

DISCUSSION

The above findings regarding the reactions of secretor semen and vaginal material are entirely empirical, but two papers published by Le Pendu et al (1982, 1983) appear to provide a scientific explanation for some of the observations. In the first paper it was demonstrated that large quantities of H type 1 and H type 2 antigenic determinants were present n the saliva of OLe^b individuals and that relatively small amounts were present in their sera. In the second paper it was shown that there were large quantities of A and B antigens in the saliva of ALe^b and BLe^b individuals respectively, but the levels in serum were not investigated.

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Interpretation of the ABH reactions of Casework Seminal Stains

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INTRODUCTION

Recently a four year study of the ABH reactions of seminal stains was completed (Davies, to be published). The stains came from cases where the identity of the assailant was virtually certain and both the ABO group and the secretor status of complainant and assailant were known. The majority of the cases involved sexual intercourse so most of the seminal stains contained at least some vaginal material. One of the more important aspects of the results of the survey was the knowledge gained regarding the success of typing different ABO groups of secretor semen, together with the possible contribution to the ABH reactions of any secretor vaginal material present.

In the following summary of the findings, where reference is made to the reactions of secretor semen of a particular ABO group, the data came from either neat seminal stains or those resulting from sexual intercourse with a female who was not a secretor of an ABO group which would contribute to those reactions. Similarly the facts regarding secretor vaginal material of groups A, B and O came from rape cases where the assailant was not a secretor of an ABO group that would contribute to the observed reactions. Very few seminal stains containing AB secretor vaginal material were grouped and these are not included in the paper.

SECRETOR SEMEN

Table 1 - The number of conclusive reactions obtained from stains of secretor semen

A secretor	B secretor	AB secretor	O secretor
67% (97/147)	66% (89/134)	64% (18/28)	40% (42/104)

The grouping of A, B and AB secretor semen was equally successful (Table 1), two-thirds of the stains gave conclusive reactions for the seminal ABO group. In contrast less than half of the stains of O secretor semen gave conclusive H reactions. (A conclusive reaction was where several dilutions of the stain extract gave strong elution reactions and a $^1/_5$ dilution gave at least 50% inhibition.)

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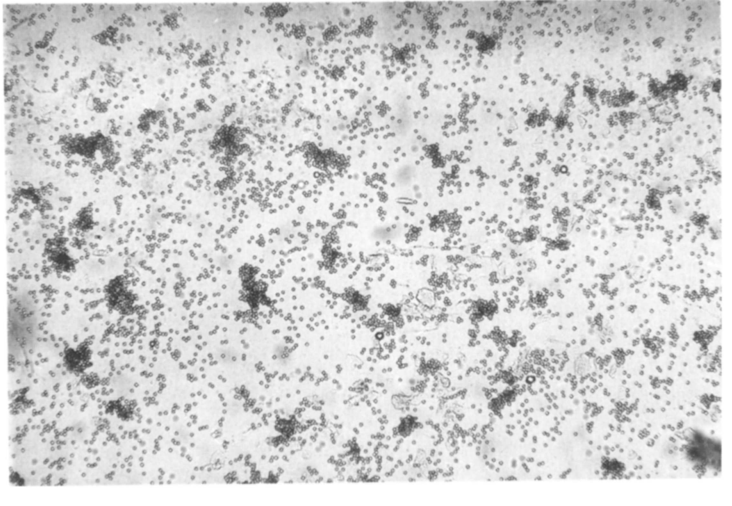
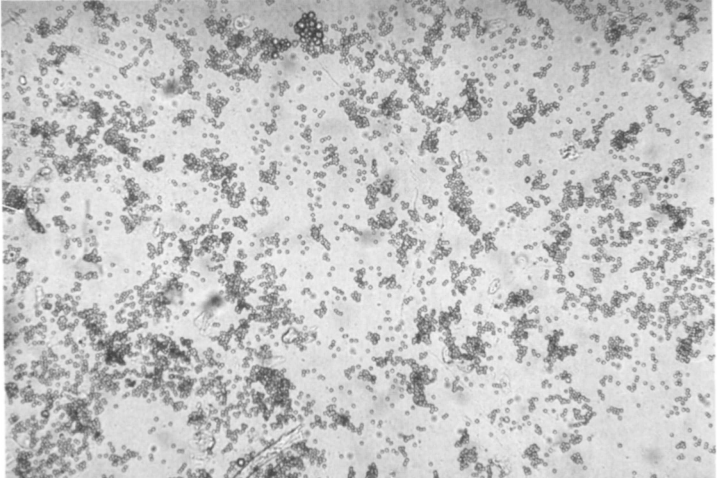
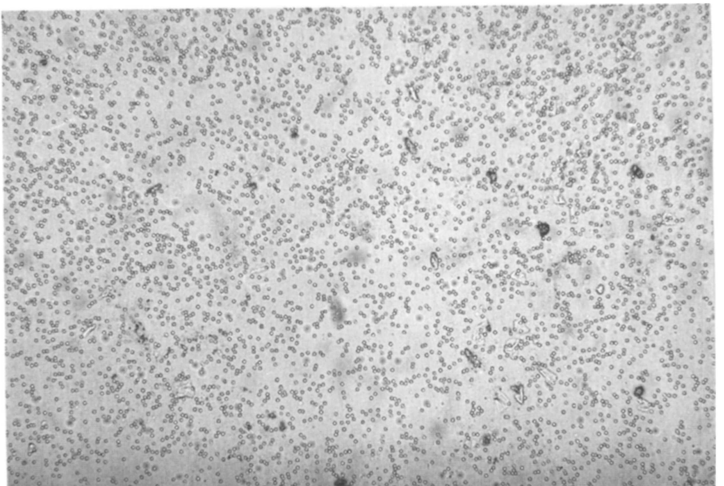


Belt
Adhesive strip
Anti-A with
A erythrocytes



Belt
Adhesive strip
Anti-B with
B erythrocytes



Belt
Adhesive strip
Anti-H with
O erythrocytes

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Blood group of victim	Blood group of suspect or perpetrator	Weapon	Result	Admission of guilt
A_1	A_1B	knife	A, B	yes
A_1	A_1B	pistol	A	suicide
A_1	B	sledgehammer	B	yes
A_1	B	sledgehammer	B	yes
A_2	B	neck of a bottle	A, B	yes
B	A_1B	knife	A, B	yes
A_1	0	knife	0	yes
B	A_1	belt	A, B	yes, see Fig.
A_2	not known	stick for strangulation	A	
0	A_1	belt	A	yes
A_2B	A_1	knife	A	yes
0	A_1	telephone cable	A	yes

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minutes) in the staining trough (with 0.9% cold NaCl solution).

After pouring off the NaCl solution carefully, allow the surface to dry and cover with a coverglass. Allow 0.5% erythrocyte suspension to run under the coverglass.

Elution at 56°C for 15 minutes, and microscopic reading after cooling.

As can be seen from the Figures illustrating the various reactions, there is pronounced agglutination of the test erythrocytes. Reading of the tests is facilitated when the cover glass is pressed slightly with a dissecting needle in microscopy. When agglutinates are present, these can then be discerned even better owing to their movement ("in swimming past") under the microscope.

Most investigation results which we obtained were retrospectively confirmed by admission of guilt on the part of the perpetrator. Cases 8 and 10 (cf. Table) involve the same perpetrator, who was caught in a further murder. A differentiation could not be carried out in only a few cases in which the murderer and victim had the same main blood group.

To summarize, the technique presented here and the results obtained can provide indications with regard to the blood group of the murderer.

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Identification of blood group substances of fingerprints on the murder weapon by means of the adhesive strip method

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Earlier investigations by ISHIYAMA, PROKOP and GÖHLER as well as KEIL and PAPSDORF (1977) have shown that it is possible to demonstrate blood group substances on fingerprints. We reported elsewhere (SIMEONI, GRÜNER and BURKHARDT 1984) on the application of the absorption elution method in the determination of the main blood group from fingerprints in excretors and nonexcretors of blood group substances.

The papillary line pattern of the perpetrator is not to be discerned on the tools with which crimes have been committed when it has been smudged, e.g. owing to substantial force. Nevertheless, a number of shed epithelial cells sufficient to allow such a determination to be carried out may be present.

MATERIALS AND METHODS

We took adhesive strip impressions from weapons which have been used in homicides using Tesafilm from Beiersdorf (Nr. 5529). In securing of evidence, it is absolutely necessary that the object concerned is grasped with disposable gloves so that no foreign cells can be transferred to it. The following weapons were investigated: knife handles, pistol grip, sledgehammer, neck of a bottle, belt, stick, telephone cable.

The following specific procedure was applied:

METHOD

Degreasing of the slide with alcohol.
Foil is stuck on to the slide evenly and free of air bubbles with the adhesive side on top using Uhu "second" glue without touching the adhesive side of the film (take care: blood group properties of the investigator).

Overlayer with two drops of antiserum
Incubation with antiserum for one hour at room temperature and for two hours at refrigerator temperature storage in a moist chamber.
Elution of the excess antiserum or lectin with 0.9% cold NaCl solution by flushing (seven times) and allowing to stand (2 x 15

*with technical assistance from H. Albin

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4. CONCLUSIONS

The new method for ABO and Lewis typing of nonblood body fluids is very easy to handle and gives stable documents after activity staining. Furthermore the high sensitivity offers the possibility of analyzing even sweat and urine stains without the need of concentrating these extracts. Last but not least there is no need of an expensive equipment.

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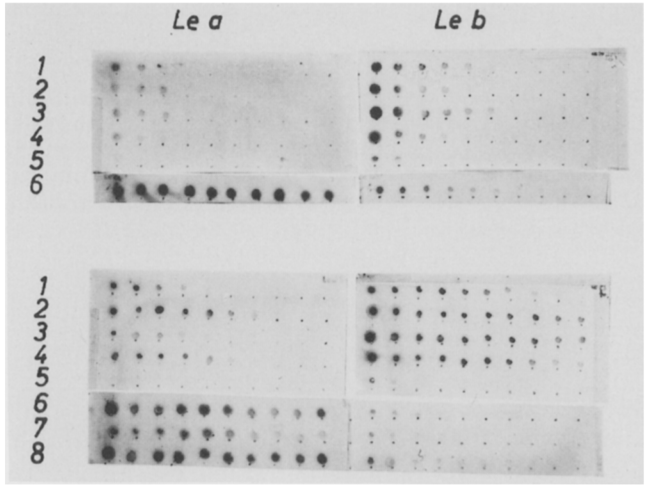

Fig. 2. Lewis typing of liquid saliva (top) and saliva stains (bottom) on nitrocellulose membranes. The ABO and Lewis status of the presented individuals and the dilution steps are the same as described in Figure 1. Two additional nonsecretor persons are added to the saliva stains (bottom, No. 7 and 8).

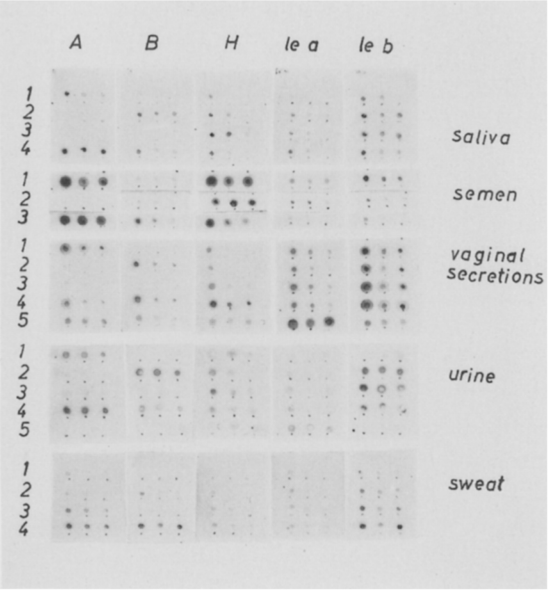

Fig. 3. ABO and Lewis typing of stains from saliva, semen, vaginal secretions, urine and sweat on nitrocellulose membranes. Persons with the following ABO and Lewis status are shown (from 1-n):
saliva: A Le Se, B Le Se, O Le Se, AB Le Se
semen: A Le Se, O Le Se, AB Le Se
vag.secretions: A Le Se, B Le Se, O Le Se, AB Le Se, AB Le se
urine: A Le Se, B Le Se, O Le Se, AB Le Se, AB Le se
sweat: A Le Se, B Le Se, A Le Se, AB Le Se
Dilution steps: 1/80, 1/320, 1/1280 for secretors (saliva, semen, vaginal secretions), 1/2, 1/4, 1/8 for secretors when typing urine and sweat and for nonsecretors.

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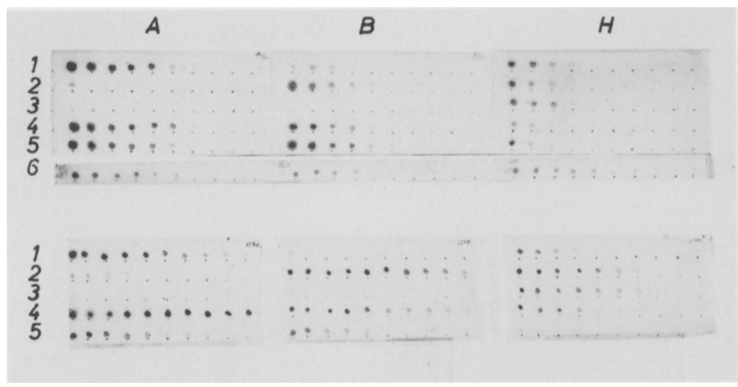

Fig. 1. ABO typing of liquid saliva (top) and saliva stains (bottom) on nitrocellulose membranes. Persons with the following ABO and Lewis status are shown (from 1 - 6): A Le Se, B Le Se, O Le Se, AB Le Se, AB le Se, AB Le se. Dilution steps for liquid saliva are fourfold, starting with 1/100 (Secretors). For saliva stains double dilution steps with an initial dilution of 1/40 are chosen. Nonsecretors are diluted 1/4 to 1/2048.

3.2 Lewis typing of liquid saliva and saliva stains

For testing Le a und Le b the same donors and the same dilution steps as described in 3.1 were used. The limits of activity for Le a and Le b varied from 5×10^{-4} to at least 10^{-5} (Fig. 2 top) for liquid saliva. Persons who are Le a-b+ by red cell typing showed considerably lower activity with anti-Le a than with anti-Le b. Individuals who are Le a+b- showed strong activity with anti-Le a and only weak activity with anti-Le b (Fig. 2 top, No. 6 and Fig. 2 bottom No. 6 - 8). A Le a-b- typed person (Fig. 2 top, No. 5) showed almost no activity. Compared with liquid saliva the saliva stains gave simil ar results in regard to sensitivity and activity distribution (Fig. 2, bottom).

3.3 ABO and Lewis typing of saliva-, semen-, vaginal secretions-, sweat- and urine stains

For each A, B, H, Le a and Le b typing we chose three different dilution steps with concentration ranges from 10^{-2} to 4×10^{-4} (saliva, semen and vaginal secretions) for secretors and 10^{0} to 10^{-1} for sweat and urine secretors and for all nonsecretors. Stain extraction was carried out with 5 % ammonium (saliva, vaginal secretions and sweat) and 6 M urea + 0,5 % BSA (semen and urine). The results we got with vaginal secretions and semen stains were comparable to those we discribed above for ABO and Lewis typing of saliva stains (Fig. 3). The ABO grouping of a nonsecretor (Fig. 3, vaginal secretions No. 5) was also possible. In addition ABO and Lewis typing of sweat and urine extracts showed also conclusive results with the exception of a nonsecretor person whose urine was only typable for Lewis but not for ABH substances (Fig. 3, urine No. 5).

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(5) Incubation with the secondary antibody. 1/400 diluted anti-mouse IgM alkaline phosphate conjugate was incubated with each of the NC's on a glass plate for 50 minutes at room temperature.

(6) washing of the membranes with washing buffer four times and further two times with washing buffer excluding Triton X-100 (each at least 5 minutes).

(7) Activity staining

To a molten solution (about 70°C) of 1,5 % agar the following solutions were added: 25 ml 0,25 M glycine/NaOH buffer, pH 10.4, 0,5 ml of 0,1 M $MgCL_2$ and 0,1 M $ZnCl_2$ and 0,1 ml of substrate stock solution. The mixture was poured on an agarose-coated polyester film (Gel-Fix for agarose, Serva, FRG), which produced a staining gel of about 240 x 70 x 2 mm.
After excess buffer was removed with filter paper the NC membranes were applied on the top of the staining agar and incubated at 37°C until dark blue dots appeared. For increasing the sensitivity, the incubation period was prolonged overnight until the agar and the membrane will be dried on the polyester sheet giving a stable document of the original results.

3. RESULTS

3.1 ABO grouping of liquid saliva and saliva stains

In tests with five secretor salivas (A Le Se, B Le Se, O Le Se, AB Le Se, AB le Se) using 0,3 µl amounts of liquid saliva with concentrations of 10^{-2} to 4 x 10^{-8} (fourfold dilution steps starting with 1/100), the limits for a minimum of detectable A, B and H activity varied from 10^{-4} (H-secretor) to 2,5 x 10^{-6} (A- and AB-secretors (Fig. 1, top No. 1 - 5). Liquid saliva of a nonsecretor (AB Le se) with double dilution steps from 1/4 to 1/2048 of pure saliva showed activity of A, B and H substances in a range of 1/32 - 1/128 (Fig. 1 top, No. 6). In comparison saliva stains from the five secretors were applied at concentrations of 2,5 x 10^{-2} to 5 x 10^{-5} by double dilution steps (Fig. 1 bottom, No. 1 - 5). The sensitivity was almost comparable with the equivalent liquid saliva, when 5 % ammonium was used for extraction. The saliva stain of the AB-nonsecretor gave no conclusive result.

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Samples

All samples were collected from donors of known ABO, Lewis and secretor type. Liquid samples (saliva) were used freshly - without any further pretreatment - with dilution steps from 10^{-2} to 5×10^{-8}. Stains of saliva, semen and urine were prepared on boiled cotton cloth. Sweat and vaginal secretions were collected on cellulose tissue. The stains were dried at room temperature and stored at -20°C until use.

Stain extraction

As reference for calculating the dilution factors we used for extraction 1 cm^2 of stained cloth equivalent to about 25 µl of neat liquid sample.
Extraction was carried out in serum samplers for about 2 h at room temperature with 250 µl (secretors) e.g. 50 µl extractant (nonsecretors), so that the neat extract was already diluted 1/10 and 1/2 respectively.

Extractants

Bidistilled water, 5 % ammonium, 6 M urea + 0,5 % BSA.

ELISA technique on Nitrocellulose (NC) (modified according to Pflug)

This is the same method as introduced for the highly sensitive detection of Gc-protein in bloodstains (Pflug 1985, Pflug 1986) after isoelectric focusing. The different steps are carried out as follows:

(1) 0,3 - 0,5 µl extract were applied with an Eppendorf Varipette on nitrocellulose membranes (0,2 µm - Schleicher und Schüll, FRG), dried at room temperature and fixed for one hour at 80°C. Prior to the application the position of each dot was marked with a pencil.

(2) blocking of free charges in the NC membranes with blocking solution by mechanical shaking for 1 h.

(3) Incubation with primary monoclonal antibody.
The optimized antibody dilutions were: Anti-A, Anti B 1/50 - 1/100, Anti-H 1/10, Anti Le a 1/2000, Anti-Le b 1/4000.
Each of the NC membranes was incubated with one of the different antibody dilutions on a glass plate covered with a plastic lid for about 50 minutes at room temperature (about 0,5 ml solution is sufficient for a NC membrane of the dimension 7 x 10 cm).

(4) washing of the membranes with washing buffer six times for about 5 minutes by mechanical shaking.

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Dot blot ELISA on Nitrocellulose membranes (NC): a new method for typing Lewis and ABO of body fluids (saliva, semen, vaginal secretions, sweat and urine)

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1. INTRODUCTION

In forensic case work Lewis and ABO typing of nonblood body fluids is currently performed by absorption-inhibition and absorption-elution techniques (Pereira et al. 1976, Davie 1979, Pinet et al. 1980, Bäßler 1986). Recently various enzyme linked immunosorbent assays (ELISA) using microtiter plates for ABO and Lewis typing were described (Lang 1985, Katsumata et al. 1984, Bolton et al. 1986). These methods are superior in respect to their sensitivity, specifity and reproducibility, but they need a high volume of stain extract and an expensive technical equipment. The work reported here describes a very simple and easy to handle ELISA method on nitrocellulose membranes which overcomes the problemes alluded to.

2. MATERIALS AND METHODS

Washing buffer

0,01 M Tris/HCl buffered saline, pH 7,4 + 1 % Triton X-100 (v/v).

Antibody diluent and blocking solution

Washing buffer + 3 % bovine serum albumin (BSA).

Antisera

monoclonal Anti-A, Anti-B (Seraclone, Biotest, FRG)
monoclonal Anti-H (Fresenius, FRG)
monoclonal Anti-Le a, Anti-Le b (Biotest or Schwab, FRG)
anti-mouse IgM-alkaline Phosphatase Conjugate (Sigma)

Substrate stock solution for alk. phosphatase staining

50 mg 5-Bromo-4-chloro-3-indolylphosphate dissolved 1 ml Dimethylformamid (stored at 4°C).

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Table 1. Absorption-elution on microplates (AEMP) and Hemagglutination Inhibition on microplates (HIM) titer in some samples of saliva and semen.-

Blood group saliva	secretors status	HIM° titer*	AEMP titer*
A	S	131072	1048576
A	S	32768	2097152
A	S	131072	1048576
A	S	1048576	4194304
A	S	524288	2097152
A	S	131072	1048576
A	NS	1024	neg
B	S	512	1048576
B	S	65536	524288
B	S	65536	1048576
B	S	32768	524288
B	S	131072	1048576
B	NS	128	neg
AB	S	A 4096	131072
		B 128	131072
AB	S	A 16384	131072
		B 512	131072
0	S	131072	131072
0	S	262144	524288
0	NS	4	neg
0	NS	64	neg
Semen			
A	S	65536	1048576
B	S	131072	2097152
0	S	131072	524288
A	NS	32	neg

° HIM: Hemagglutination Inhibition on U Microplates; Seraclone A diluted 1:400, Seraclone B diluted 1:200, anti-H diluted 1:50.
* titer: dilution end-points.

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A secretor, 5 mm side, extracted with 0.5 ml saline, was 1/2000 with HIM, and 10 µL of the extract had titer of about 1/500.000 with AEMP.
Negative findings with nonsecretors (which are positive with the conventional absorption-elution tests directly performed on the stain) are presumably due to ABH substances different in molecular size and shape, that probably link to the plate but in such a way that the antibody is hindered from binding or bind in non eluable form.

In forensic casework AEMP can be conveniently used in combination with Hemagglutination Inhibition on 'U' Microplates (HIM) with monoclonal antibodies and with the direct conventional Absorption-Elution (AE) to check the blood group and the secretor status, nonsecretors being positive with AE and often with HIM, usually negative with AEMP.

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The wells were empted and washed three times, each of 10 minutes, with the washing solution.
One hundred uL blocking solution was then added to each well and left for 30 min. at room temperature.
The plate was shaken dry and washed again, three times, with saline. Then each well was filled with 50 µL anti-A or anti-B polyclonal antibody (Ortho Diagn.) diluted 1:3 with the diluent. Anti-H from Ulex Europaeus (titre 1/128) was used undiluted. Monoclonal anti-A and anti-B were also assayed (Seraclone, Biotest; Bioclone, Ortho)
After 3 hours incubation at room temperature, the plate was washed twice with washing solution, then rinsed with tap water for 10 minutes, and finally with saline.
Fifty µL diluent was added to each well, the plate was covered and elution carried out for 1 hr in an oven at 60°C. Equal volumes 0.5% indicator red cells were added and sedimentation allowed to occur at room temperature for 3-4 hrs. The plate was then preserved at 4°C to be reexamined.
Macroscopic reading of the agglutination patterns was performed as elsewhere described for 'U' plates (Fiori, 1985). Accordingly, strong agglutination is seen as a small uniform salmon-pink disc covering the entire lower part of the wells, and partial agglutination as rings with increasing thickness and decreasing diameter. Complete inhibition is a smooth round button that forms a tear when the plate is inclinate in semi-vertical position for some minutes.
Haemagglutination Inhibition on 'U' Microplates (HIM was carried out by using diluted monoclonal anti-A and anti-B (Seraclone, Biotest) and anti-H from Ulex Europaeus according to the method described elsewhere (Fiori 1985).

RESULTS AND DISCUSSION

Liquid samples of saliva and semen of A, B and AB secretors gave highly sensitive results at dilutions ranging from 1×10^5 to 5×10^6. In 0 secretors the positive results were usually in the range of 1×10^5 to 6×10^5.
In Table I some examples are reported. Nonsecretors were in most cases negative. Only in some specimens a few wells showed partial ed irregular agglutinations.
Monoclonal antibodies gave always negative results as in MASP method. Nonspecific agglutinations with heterelogous sera were not observed when the whole procedure was carefully followed, especially the washing and blocking steps.
The stain extracts were positive at lower dilutions (in general up to 4×10^4 - 8×10^5) apparently due to the incomplete elution from the stain. But sometimes the results were of high sensitivity. For example a seminal stain from a

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Absorption-elution on microplates (AEMP): An improved method for identification of th ABH major glycoproteins in saliva and semen of secretors.

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INTRODUCTION

Recent progress in ABO typing of body fluids and stains have been obtained by highly sensitive methods such as Inhibition of Hemagglutination on Microtiter Plates (HIM) by using monoclonal antibodies (Fiori, 1985), enzyme-immunoassays (EIA) as the ELISA procedures (Inoue and Okada, 1978; Fletcher 1984; Bolton and Thorpe, 1986; Mudd 1986; Mukoyama and Sakai 1986; Takatori and Tsutsubuchi 1986), and EIA-like Mixed Agglutination on Solid Phase (MASP; Fiori and Chiarotti, 1985, Silvestri et al. 1981).
The ELISA procedures are expensive and time-consuming, MASP is tedious and poorly reproducible. Therefore a new method was devised, Absorption-Elution on Microplates (AEMP), that does not require apparatus and is as sensitive (if more) as ELISA (FIORI, 1985). This method is based on the linkage of soluble ABH substances to the bottom of 'U' wells of polystyrene microtiter plates, with subsequent absorption of polyclonal antibodies, elution by heating and detection of the eluted antibodies by microagglutination of indicator red cells.
In the present paper an optimized version of the AEMP is presented.

MATERIALS AND METHOD

Saliva and semen samples from A, B and O secretors and nonsecretors were examined after centrifugation and without any denaturating treatment. Small cloth pieces (2 x 5 mm) stained with saliva and semen were extracted overnight in 0.1-0.2 ml saline.
The coating solution was 0.05 mol/L carbonate/bicarbonate buffer pH 9.6; the diluent for red cells, antibodies and the eluate medium was saline containing 0.2% (w/v) BSA.
The washing solution was saline containing 0.1% gelatin and 0.05% Tween 20; the blocking solution was saline containing 0.2% (w/v) BSA and 0.1% Tween 20.
Samples (50 μL each) were serially diluted in 24 wells with the coating buffer and incubated overnight at 4°C.

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Cell contaminated sections: The blood grouping of red blood and buccal cells sticking to the nail surface revealed encouraging results. To illustrate this, we were able for instance to distinguish between group A erythrocytes or group B buccal cells on the one hand and group O of the layer of nail on the other hand. We have to stress, that in general more complicated constellations such as group AB materials on fingernails of group O cannot result in a diagnosis without restrictions.

In general, positive reactions are weak in comparison with other tissue material because of the low concentration of ABH antigens in nails. The drawbacks of all immunoenzyme methods are their numerous reaction stages with extended incubation times. In our experiences the incubation of nail-keratin with primary antibody should not be reduced below 48 hours. Considering the total of working steps the test needs more than 3 days. As a rule, we have to expect an additional damage of stain material from the scene. Therefore, the prior storage time for red blood and buccal cells should not exceed a few days only. This limitations does not concern pure fingernail samples.

CONCLUSIONS

1) APAAP staining is a proper method for demonstrating blood group antigens in fingernails from groove to margin.

2) Frozen sections as well as smallest specimen embedded in a suitable adhesive are applicable for staining procedures.

3) Using fresh prepared artificial stains, blood group constellations of red blood cells and/or buccal cells adherent on the surface of fingernails may be distinguished from the nail matrix.

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APAAP staining: The following commercially available reagents were used:

Monoclonal mouse, anti-A, anti-B, anti-H (Seraclone) - Biotest
Rabbit anti-mouse immunoglobulins - Dakopatts
Alkaline phosphatase-anti-alkaline phosphatase complex(mouse) - Dakopatts
Naphtol AS-MX phosphate - Serva
Fast Red TR - Serva,Chemapol
Fast Blue RR - Chemapol

All specimen were washed in Tris-buffered saline (0.05 M, pH 7.6) before each reaction stage. Monoclonal anti-A,anti-B and anti-H were diluted 1:50 and incubated with the samples in a moist chamber for 72h at +4°C. The incubation with the linking rabbit anti-mouse antibody (dilution 1:25) was carried out overnight at +4°C. The APAAP complex (dilution 1:300) were coupled for 2 hours at room temperature. The visualisation of the APAAP complex was achieved by treating the reactants with the appropriate dye for 30 min.. Red colouring were intensive when positive. The blue dye, however, allowed a better distinction of positive red blood cells from negative erythrocytes with their individual reddish colour.

Controls: Positive and negative controls must be processed along with all unknown specimen. A nonspecific staining due to endogenous alkaline phosphatase or protein binding was excluded by blank controls with buffer. For testing the specifity of the primary ABH antibody we controlled the lack of reactivity of incompatible antibodies in each case, e.g. anti-B against group A etc.. The reliable and specific tissue staining of epidermis belonging to specimen has proved an excellent tool of control for ABH determination in nail-keratin.

RESULTS AND DISCUSSION

Frozen sections: Frozen slides are the specimen of choice. The adherent nail-bed epidermis allows a clear-cut determination of ABO constellations in the stratum germinativum. The validity of ABO grouping decreases in our material in the known rank order: B> A> H> AB. As expected, we found a weak background colouring of nails after anti-H incubation. However, the striped and dotted colour pattern of keratin tissue enabled the specific staining reaction to be recognized. Thorough inspection of the whole specimen is necessary.

Adhesive embedded sections: In practice the investigator is confronted with small nail-fragment samples, which have to be prepared as slides in a suitable embedding medium. In accordance with GRIEVE and KOTOWSKI (1986) we found that the adhesive UHU meets all criterias for our purposes: It allows easy sectioning without being so hard as to brittle and chemically it is inert. The determination of A and B substances was also succesful in all samples. Nevertheless, nonspecific staining may occur at the border to the embedding medium.

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Determination of ABH antigens in fingernails using the APAAP (immunoalkaline phosphatase) technique

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In contrast to many investigations into the presence of ABH antigens in human hairs the reports on the blood grouping of fingernails are rare (YADA et al.1966; KIRST et al.1971). By means of absorption/elution technique the presence of blood group substances was demonstrated without attaching to known morphologic structures of layer of nail. Fragments of fingernails may become traces themselves or stain carrier in forensic cases. Therefore, the direct visualisation of ABH pattern in nail-keratin and the discrimination of adherent blood group substances will be important.

The introduction of immunoenzyme methods in different modifications allows investigations on the localisation of blood group substances in cells and tissues from histologic slides (PEDAL and HÜLLE 1984; LÖTTERLE and HEINE 1986), hairs (PÖTSCH-SCHNEIDER et al.1986) and body fluids (BRINKMANN et al. 1986). We applied the APAAP technique for two reasons:
1) There are versatile possibilities of specimen colouring.
2) The inhibition of endogenous peroxidase by hydrogen peroxide can be omitted.

MATERIALS AND METHODS

Specimen preparation: Fingernail specimen with adherent nail-bed were taken from autoptic material of 8 corpses with blood groups A, AB, B and O. Frozen 4-5 µ sections were submerged and floated carefully during each working step.Portions of fingernails were contaminated with blood and buccal cells, respectively. The adherent cells were fixed by aceton/methanol(1:1). A limited number of nail samples were prepared with blood/buccal cell mixtures in various ABO constellations. For saving sera and reagents we had to use defined stain material. Blood grouping of mixed material requires a directed regimen of repeated incubations with anti-H, anti-A and anti-B antibodies. The microtome-section technique, however, permits the production of a sufficient number of specimen for smallest stain samples.

Furthermore, fingernail fragments of 8 volunteers were embedded in a biocomponent adhesive (UHU plus schnellfest(R)) according to GRIEVE and KOTOWSKI (1986) and cut in the usual microtome technique.

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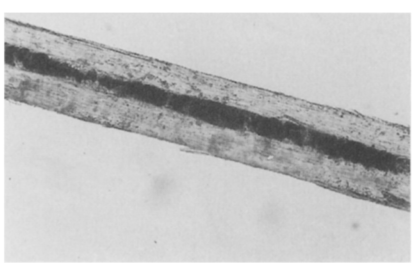

Fig. 1. Positive staining of medulla in a hair from group A_1 individual tested with anti-A.

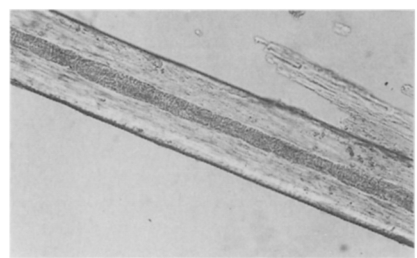

Fig. 2. Negative staining of medulla in a hair from group A_1 individual tested with anti-B.

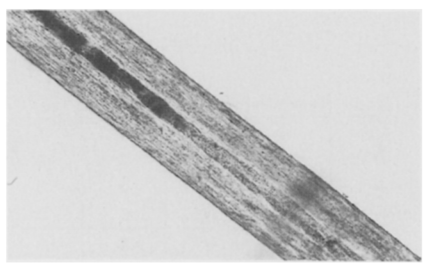

Fig. 3. Mosaic staining pattern of medulla in hair from group A_1 individual tested with anti-A.

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in the hands of other groups.
One obvious drawback is the wide variation in the presence and type of medulla in caucasians. A microscopical examination of the questioned hair must therefore precede any blood grouping investigation using this method.
The location of A, B and H antigens only in the medulla could explain the discrepancy between previous inconclusive studies where the results obtained were possibly dependant on the presence or absence of a medulla and not on the blood group of the individual.

The observed mosaic distribution of the antigens within the medulla and independance of secretor status can also be observed in other organs and tissue structures of the body. Several theories have been put forward as to their origin and genetic development and it remains to be seen whether the studies on hairs will throw any light onto the now seemingly complicated distribution of the A, B, H and lewis antigens in the human body.

ABO Grouping of Hairs: Avidin-Biotin

Blood	Number	Correct	False	Weak
A_1	15	15	0	0
A_2	5	3	0	2
B	6	6	0	0
O	15	15	0	0
A_1B	5	4	0	1
A_2B	1	0	0	1
Total	47	43	0	4

Table 1a. ABO grouping results of 59 post-mortem hair samples using the Avidin-Biotin technique. 12 samples were not tested because they possessed no medulla.

Distribution of Blood Groups

Blood	Number	Correct	False	Weak
a- b+	28	27	0	1
a+ b-	7	4	0	3
a- b-	5	5	0	0
Unknown	7	7	0	0
Total	47	43	0	4

Table 1b. ABO grouping results from Table 1a in comparison to the distribution of Lewis groups in the sample.

ABO Grouping of Hairs: Blind Trial

	Number	Correct	False
A_1	10	9	0
A_2	2	1	0
B	1	1	0
O	15	11	0
Total	28	22	0

6 samples possessed no medulla

Table 1c. ABO grouping results of 28 hair samples in a blind trial. 6 samples were not tested because they possessed no medulla.

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MATERIALS AND METHODS

The test sample consisted of 59 post-mortem hair samples from individuals ranging from 5-60 years old and post-mortem intervals ranging from 1 day to 6 months. In addition a blind trial of hairs from 28 volunteers was carried out. The technique used was similar to that employed by Pötsch-Schneider except that A, B and H monoclonal antibodies in conjunction with biotinilated rabbit anti-mouse antibody and the sensitive Avidin-biotin-peroxidase complex was used. The hairs were longitudinally sectioned by hand before being treated as described and finally embedded and examined microscopically.

RESULTS AND DISCUSSION

The results are listed in tables 1 (a-c) and as can be seen no false positive results were found, thus substantiating the claim made by the previous investigators.Figures 1,2 show typical positive and negative results obtained during this study.

However the following precautionary comments should be made.

1. As confirmed by all the studies, the antigens were specifically demonstrated but only in the medulla of the hairs. The presence of a medulla varies between races, and can also show inter- and intra-individual variation. The form can range from complete absence through intermittent, broken to continuous so that the necessity of first locating the medulla, or hairs which possess a medulla, must be strongly stressed.
2. The presence of a medulla does not necessarily guarantee a 100 % reaction: several samples were found during the study which showed a mosaic pattern of distribution of staining (fig. 3). The method of longitudinal sectioning would seem to most minimise the risk but when cross-sections are examined care must be taken not to concentrate on too small an area.
3. Completely negative results were found but only in two cases where the body had been in sea water for 6 months. It does however demonstrate the necessity to test for all three A, B and H antigens as is the practice in all other forensic applications of the ABO system.

SUMMARY

A, B and H antigens have been demonstrated with complete success in three independant studies using immunohistochemical techniques and would appear to have a practical application in forensic science. In view of other previous claims of success many more series of tests must obviously be carried out to demonstrate not only the accuracy but also the reproduceability of the results

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Immunochemical detection of ABH antigens in hairs

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INTRODUCTION

The development of new techniques in one field of science has always lead to a proliferation of applications in vastly different areas of research and applied science. This is especially noticeable where the previously used methods have proved to be unreliable or inadequate for the job.

It is not surprising therefore, that the immunohistochemical method first reported in 1979 by Sternberger for the identification of spirochaetes has since been modified and been extensively applied to problems in forensic science. In particular it seems to have found an application in the problem of the detection of A, B and H antigens in hairs.

Since the first recorded reports from Sakai in 1951 and later from the many reports from Yada and his colleagues, the presence of A, B and H antigens in hairs has been extensively studied and the results discussed in depth.

The 1oo % success rate claimed by the japanese workers has never been clearly demonstrated by any of the numerous other groups of workers. Methods ranging from the Absorption-Elution method used by Yada, mixed agglutination by Lincoln (1968), radio-active labelled antibodies by Boettcher (1973) and many others have been employed, but have always produced a quota of false results especially with respect to blood groups A and O.

The first application of the immunohistochemical method was successfully carried out, again in Japan by Miyasaka (1984 and 1987) and also by Yoshida (1984). This was followed by the report from Pötsch-Schneider (1986) who reported correctly determining the blood group of hair samples from 168 individuals using the PAP and APAAP techniques. Specific staining was demonstrated in the medulla of hairs and was found to be independant of secretor status.
In view of previous claims of success this study was carried out to see if the results could be successfully reproduced.

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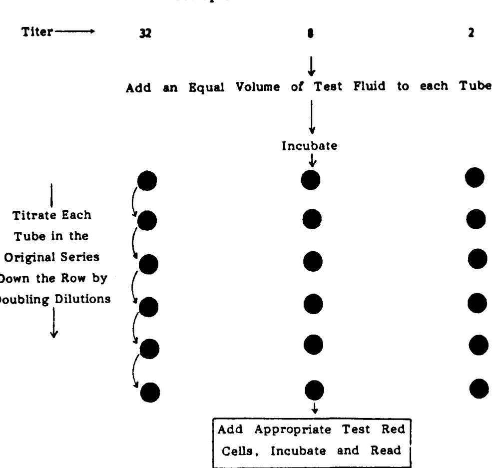

Fig. 2. Two Dimensional Procedure for Casework Using Selected Dilutions of Antisera

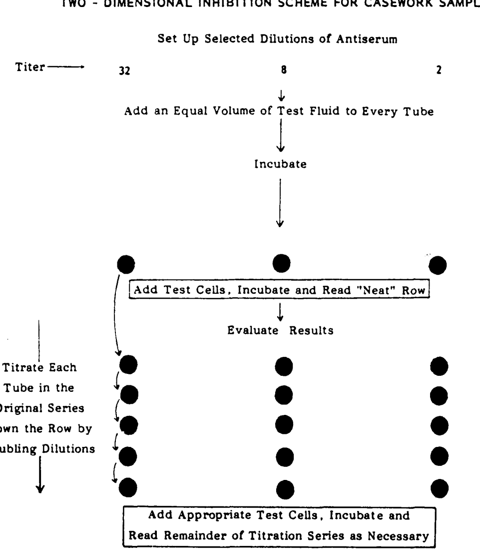

Fig. 3. Two Dimensional Procedure Scheme for Casework in which Results of the TI Dimension are Read Before Proceeding to the IT Dimension

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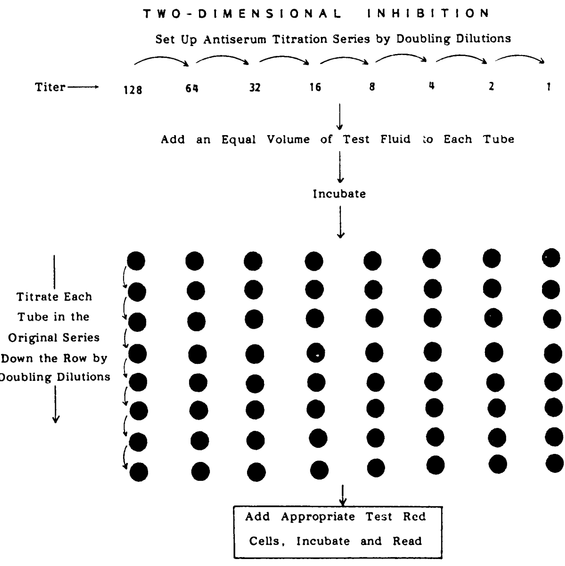

Fig. 1. Overall Two Dimensional Procedure

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The recent increase in drug screening tests on individuals using urine has made the reliable typing of urines as an aid to identification more important. Urine samples thought to have been mislabeled or incorrectly tested for drugs are now often submitted with a request to compare the grouping results with the ABO group and secretor status of the alleged donor of the urine.

In casework, however, even stains of saliva and semen are sometimes difficult to group correctly by conventional techniques, either because their soluble antigen content is intrinsically low or because the quantity of sample available is low, or both. Table 2 shows that in a number of these cases, the 2D technique has enabled us to obtain conclusive results which would have been unobtainable without it.

Table 2. Results Obtained in Problem Case Samples with the Four Inhibition Techniques

Type of Sample	ABO Group and Secretor Status	Results Obtained With One Tube	I-T	T-I	2-D
Saliva	A secr	H	(A),(H)	A,H	A,H
	AB secr	B	(B),(H)	(B),(H)	A,B,H
	AB secr	A	(A),(B)	A,B,H	A,B,H
Semen	AB secr	A,H	A,(B),(H)	A,B,H	A,B,H
	A secr	H	(A),(H)	(A),(H)	A,H

( ) = weak, inconclusive results for the antigen

The 2-D procedure thus provides an additional measure of sensitivity that is especially valuable in resolving the ABH antigen content of samples having ABH antigen concentrations too low to be reliably detected by existing procedures. We have used the technique in conjunction with absorption-elution as a reliable method for grouping bone tissue (see Lee and Gaensslen, these Proceedings). If the procedure suggested in Fig. 3 is followed, the 2-D technique need only be carried to completion when the IT test represented by the first row does not provided conclusive results. This approach conserves valuable examiner time and effort, and thus enables a laboratory to use this technique routinely.

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Table 1. Results Obtained on Known Control Samples with the Four Inhibition Techniques

Type of Sample	ABO Group	N	Incorrect (X) or Inconclusive (INC) Results Obtained Using: One Tube X	One Tube INC	I-T X	I-T INC	T-I X	T-I INC	2-D X	2-D INC
Secretor	A	13	1	na	0	2	0	0	0	0
	B	7	1	na	0	1	0	0	0	0
Salivas	O	9	2	na	0	1	0	0	0	0
	AB	7	3	na	1	3	0	0	0	0
Secretor	A	2	1	na	0	1	0	0	0	0
	B	2	0	na	0	0	0	0	0	0
Semens	O	3	0	na	0	0	0	0	0	0
	AB	2	1	na	0	1	0	0	0	0
Secretor	A	19	6	na	5	7	0	1	0	0
	B	15	2	na	0	5	0	1	0	0
Urines	O	15	7	na	1	5	0	1	0	0
	AB	11	4	na	1	2	0	1	0	0
Secretor	A	11	5	na	2	2	0	0	0	0
Urine	B	16	4	na	0	3	0	1	0	0
Stains	O	10	4	na	2	2	0	0	0	0
	AB	6	5	na	4	1	0	1	0	0
Secretor	A	12	8	na	3	4	0	0	0	0
Sweat	B	10	5	na	0	3	0	1	0	0
Stains	O	10	6	na	2	2	0	0	0	0
	AB	7	3	na	2	0	0	0	0	0

na = not applicable

The value of the increased sensitivity of the 2D procedure as compared even with TI, however, is seen in the results obtained for urines and perspiration. These fluids, as is well known, typically have significantly lower concentrations of soluble ABH antigens than the corresponding saliva or semen of secretor individuals. Here, IT gave a number of incorrect results. And although no incorrect results were obtained with TI, a number of samples yielded inconclusive results. The 2D procedure by contrast resolved all of these inconclusives.

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The two dimensional procedure described here takes maximum advantage of the best features of both TI and IT, and can be shown to be more sensitive than either TI or IT alone.

METHODS

The two dimensional absorption inhibition procedure consists of first constructing a TI test protocol, then treating each tube in the row as though it were the first tube in an IT test. The complete test protocol is illustrated in Fig. 1 with an initial antiserum titer of 1:128. In practice, a lower initial titer (usually 1:32) is used. Further, the test can be carried out using three or four selected antiserum dilutions without sacrificing sensitivity, which is the technique's principal advantage (Fig. 2). This approach simplifies the protocol and reduces the time required to carry out the test in routine casework. An additional simplification involves setting up the first row with two volumes of sample and antisera, then removing two volumes to another row for possible titration in the second dimension (Fig. 3). Test cells are then added to the original tubes and the results read (essentially a TI test) before proceeding. If the results are conclusive, it is not necessary to carry out the remainder of the procedure.

RESULTS and DISCUSSION

The two-dimensional (2D) absorption inhibition procedures has been used to test a number of known control secretor body fluids and stains of potential forensic interest including 36 salivas, 9 semens, 60 urines, 43 urine stains and 39 perspiration stains representing all four ABO blood groups. The tests were run in parallel with the other three established procedures for carrying out inhibition tests. Donors of the known control samples were classified as secretors on the basis of TI tests on their saliva. Table 1 shows the results of the comparative study. A result is classified as "incorrect" if the test failed to detect one or more antigens known to be present and where this failure would have resulted in the incorrect classification of secretor status or in an incorrect classification of the ABO group of origin. A titration technique result is classified as "inconclusive" if it failed to give convincing reduction of the antibody content relative to the saline control (less than three tubes).

Many incorrect results with all samples were recorded using the one tube technique. By its nature, the procedure cannot be expected to yield reliable detection of soluble antigens in secretor body fluids which are known to have a wide range of concentrations of the blood group substances. With saliva and semen, IT gave no incorrect results but seven salivas and two semens yielded a inconclusive results. TI gave uniformly correct results with these samples, and no inconclusives, illustrating the greater sensitivity of TI as compared with IT. And, for the saliva and semen samples tested, TI was as effective as 2D.

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Typing of ABH Antigens in Body Fluids by a Two Dimensional Absorption-Inhibition Procedure

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INTRODUCTION

Absorption-inhibition procedures have been used for many years for determining the ABH antigens of dried blood and body fluid samples (Gaensslen, 1983). Although the absorption elution technique originally devised by Siracusa (1923) and refined by Kind (1960) is now used almost exclusively for ABH antigen typing of bloodstains, inhibition remains the method choice in many laboratories for determination of the soluble ABH substances in secretor body fluids and body fluid stains.

There are three categories of inhibition techniques in common use: the one-step or all-or-none; inhibition titration (Holzer, 1937); and titration inhibition (Hirszfeld and Amzel, 1932; Kind, 1955). The sensitivity of the one-step procedure is inversely related to the titer of the antisera used for testing. The titration methods have been adopted in a number of laboratories to try to give a more conclusive indication of the antigen content of questioned samples given the range of variation of ABH antigen concentrations in different secretor body fluids, and the range of variation of concentrations of these antigens in stain extracts. The inhibition titration (IT) and titration inhibition (TI) procedures differ in sensitivity. One way of demonstrating these differences is examining the types of agglutination results one would expect to obtain under test conditions where sufficient antigen is present in the sample to remove a defined quantity (1/2, 1/4, 1/8, etc.) of available antibody. When this analysis is done, it can be shown that TI is more sensitive than IT. In a five tube test with an initial antiserum titer of 1:32, for example, a quantity of antigen sufficient to bind half the available antibody would result in a one tube reduction using IT, but would result in a four tube reduction using TI, as compared with the saline control row. A number of workers prefer to observe a three or more tube reduction with a titration technique before considering the result conclusive. The smallest quantity of antigen in a sample that will still give a three tube reduction by TI is an amount sufficient to bind 1/4 of the available antibody. This same quantity of antigen would yield no inhibition in an IT test, and it would give ambiguous results at best in a one step test.

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different reagents, one labelled with each fluorochrome, can be performed simultaneously on the same tissue section.

Fluorochrome	Excitation	Emission
AMCA	U.V.	blue
FITC	blue	green
TRITC	green	red

Since, with each fluorochrome, only positively labelled structures fluoresce against a black background, two or three exposures of each field, each with a different filterset, can be superimposed on the same picture. These double or triple exposures give polychromatic pictures showing the location of 2 or 3 different antigens on the same slide (Oriol et al. 1985, Mollicone et al. 1986, Le Pendu et al. 1986, Oriol 1987).

The final result can be further improved by specific yellow fluorescent staining of the DNA of nuclei. This is obtained by mounting the stained histological preparations with one drop of p-phenylenediame (1mg/ml in glycerol 90% pH 8) (Oriol and Mancilla 1983). This mounting medium enables the nuclei of all cells to be visualized in yellow with the same filterset used for FITC. In addition, it decreases the photobleaching effect undergone by fluorescein under light exposure and allows shorter exposure times.

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normal rectal mucosa (Wiley et al. 1981). However, the Le^a antigen persists thoughout the colonic mucosa and other Lewis related antigens (of as yet unidentified structure) appear in this area (Macartney et al. 1986).

Kidney. This organ contains structures from two embryonic origins. Glomeruli and proximal and distal convoluted tubules derive from the local mesenchima and express ABH and related antigens independent of *Se* and *Le* genes, whereas the urinary epithelium (collecting ducts, calyces and ureter) derive from the ureteral bud which has its origin close to the cloaca of the primitive digestive tube. These cells express ABH antigens under the control of *Se* and *Le* genes. Nevertheless, although all kidney cells follow these general rules, each portion of the nephron has its own particular expression of ABH and related structures. The endothelial cells of glomeruli and vessels and the epithelial cells of some distal tubules express ABH antigens independent of *Se* and *Le* genes (Hinglais et al. 1981). The epithelial cells of proximal convoluted tubules and the descending limb of the Henle loop are devoid of ABH antigens, but they contain large amounts of the X and Le^x antigen. This X antigen is probably under the control of the same ***X*** gene controlling the synthesis of the α-3-fucosyltransferase in serum since the proximal convoluted tubules of a young female, whose serum was devoid of α-3-fucosyltransferase, did not contain any detectable X antigen. The ascending portion of the Henle loop and the distal convoluted tubule secrete the Tamm-Horsfall glycoprotein. Finally, the Lewis antigens, under the control of the product of the Lewis gene, are only found in some distal convoluted tubules and the urinary epithelium (Oriol et al. 1980).

Type 3 antigens. Type-3 antigens contain an internal αGalNAc, which is the terminal structure of A antigens. Therefore, they have been described in A individuals as repetitive A structures and they contribute to the A_1-A_2 difference on red cells (Clausen et al. 1985, 1986). They have been found in all cells expressing A antigens, although in nucleated cells they are restricted to the area of the Golgi appararus (Le Pendu et al. 1986). The fact that the anti-A type-3 antibodies only stain the Golgi apparatus suggests that the terminal portion of type-3 structures might either be cut off, by specific glycosidases, or masked by the elongation of the oligosaccharide chains during their transit through the Golgi cysternae.

Type 4 antigens. An A type 4 heptaglycosylceramide has been isolated from human kidneys and represents the major blood group related glycolipid structure of this organ (Breimer and Samuelsson 1986).

Type 5 and 6 antigens. They have been found in exocrine secretions like milk or urine. Type 6 chains are probably the core of the ABH antigens found in the gastric surface epithelium of nonsecretors.

POLYCHROMATIC IMMUNOFLUORESCENCE

ABH and related antigens were located on tissues with specific antibodies or lectins labelled with one of the following three fluorochromes: a. fluorescein isothiocyanate (FITC); b. tetramethyl rhodamine isothioryanate (TRITC); c. amino methil coumarin acetic acid (AMCA). This last fluorochrome has been recently commercialized by Bio-Carb (Khalfan et al. 1986). All three fluorochromes can be used with the same light source, but each has different excitation and emission optima. Therefore, specific filtersets must be used to visualize each fluorochrome, but reactions with the three

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Tissue distribution for these two kinds of genetic control of ABH and related antigens is not random. Tissues of ***ectoblast*** and ***mesoblast*** origin express mainly ABH antigens independent of control by *Se* and *Le* genes, whilst tissues of ***endoblast*** origin express mainly ABH antigens under the control of ***Se*** and ***Le*** genes. However, this distribution is not an all or none phenomenon and some exceptions to this general rule have already been found (Oriol et al. 1986) .

TISSUE ABH ANTIGENS IN ADULTS

In man, the most widely distributed ABH antigens, are mesodermal type-2 antigens of erythrocyte and vascular endothelium independent of ***Se*** and ***Le*** genes. In fact, these structures are present all over the human body.

Epidermis. The human body is covered by a continuous two to four cell layer sheet of ABH positive cells which are independent of the *Se* and *Le* genes. This layer forms the most external area of the stratum granulosum, next to the stratum corneum. The only skin sample negative with anti-A, anti-B and anti-H, belonged to an H deficient individual ***(h/h)*** from Reunion Island (Le Pendu et al. 1986), suggesting that epidermal ABH antigens are under *H* locus conrol.

Primary sensory neurons. The pseudo-unipolar neurons of the posterior root ganglia have ABH antigens in the Golgi cysternae, membrane and cytoplasm. This positive reaction extends centrally to the first synapsis in the substantia gelatinosa of the posterior horn of the spinal cord (laminae II) and peripherally to the sensory receptors. Neurons of sympathic and parasympathic ganglia (Oriol et al. 1984) and the neurons of the mesencephalic nucleus of the trigeminal nerve (Mollicone et al. 1986a) are also positive. Some of the primary sensory neurons of the cranial nerves also synthesize ABH antigens i.e. olfactory, auditory (Mollicone et al. 1985a) and taste receptors. All these sensory cells expressing ABH antigens are derived either from the neural crest or ectodermal placodes. By contrast, sensory cells derived from the central nervous systems, for example the optical receptors of the retina, have no ABH antigens at all. Epithelial and endothelial cells in the cornea, which have no sensory function but are derived from the ectoderm directly overlying the optic vesicle, express ABH antigens (Salisbury and Gebhardt 1981). All ABH antigens expressed by primary sensory neurons are independent of *Se* and *Le* genes, as expected for ectodermal structures.

Digestive mucosae. This is a typical tissue of endodermal origin. All digestive epithelial cells from oral to anal mucosa express some sort of blood group related oligosaccharide antigen. Most of them express ABH and Lewis antigens under the control of *Se* and *Le* genes. However, some exceptions to this general rule have been observed in acinar cells of gastric and Brünner's glands. The surface epithelium of the duodenum and the pylorous express type 1 and type 2 ABH antigens under the control of *Le* and *Se* genes (Mollicone et al. 1985b and 1986b). By contrast, in deep gastric glands and Brünner's glands, only type-2 difucosylated antigens independent of *Se* and *Le* genes are found. These deep glands, therefore, represent an exception to the endodermal pattern of ABH antigen secretion. The remainder of the small intestine expresses ABH and Lewis antigens under the control of *Se* and *Le* genes, as does the surface of the pyloric and duodenal mucosae. The caecum and the ascending and transverse portions of the colon express these antigens under similar genetic controls. In the mucosa of the descending and sigmoideum colon, there is a progressive decrease in ABH and Le^b antigen expression. These antigens are practically absent from

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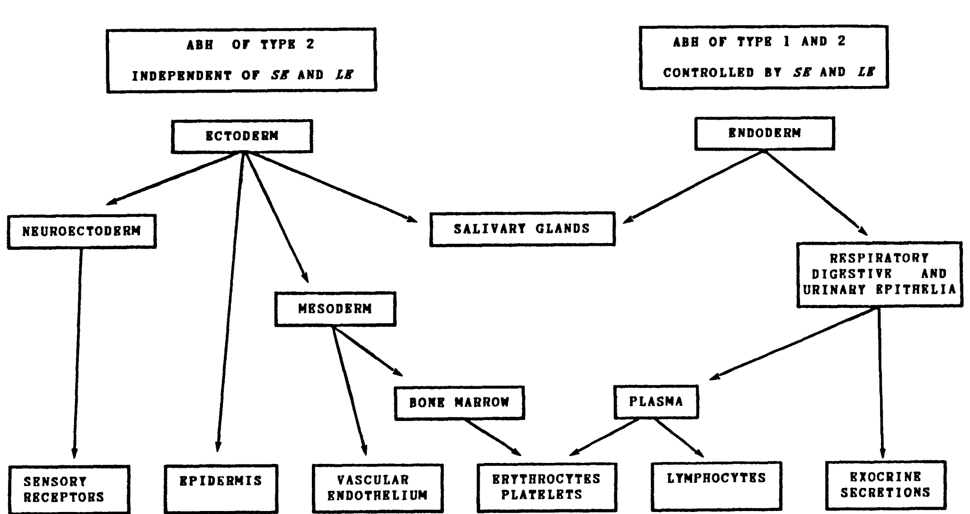
ONTOGENY

ABH antigens have been found in the earliest ascertainable stages of development of the human embryo (5th week postfertilization). At this early stage, red cells, vascular endothelium and epithelial cells of practically all organs or organ rudiments express ABH antigens in accordance with embryo ABO blood group (Szulman 1980).

The end of the first trimester of pregnancy is marked by orderly recession of the epithelial cell wall ABH antigens of many primitive organs. Their disappearance or decrease often coincides with recognizable steps of tissue differentiation. For exemple, secretion of mucus in the gastrointestinal mucosae, transition from a solid epithelial crescent to an organ with acini in the thyroid, production of growth hormone in the anterior pituitary gland are all developmental steps related to a strong decrease in expression of ABH antigens in each of the above mentioned organs (Szulman 1964). However, this recession is not uniform in all organs. The adult human rectum has completely lost the capacity to secrete ABH antigens at both cell membrane and mucous secretions, whereas upper portions of the digestive mucosa lose part of the cell wall ABH antigens but keep the mucous ABH antigens throughout life. Red cells and vascular endothelium, by contrast, do not modify the expression of ABH throughout life.

Based on the chemical structures of the ABH antigens isolated from different tissues and their expression on individuals with different genetic backgrounds, two main kinds of genetic control of ABH and related antigens have been found in man: I. ABH antigens of type-1 and type-2 under the control of the *Se* and *Le* genes. II. Type-2 ABH antigens independent of *Se* and *Le* gene control. These tissue antigens are expected to be under *H* and *X* gene control (Figure 2).



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digestive group polymorphisms, A-O and Y-O, which segregate independently (Oriol, unpublished results).

Marmoset. This small South American monkey belongs to the most primitive infraorder of higher primates, the *Platyrrhini*. All the individuals tested had A and H antigens in exocrine secretions and primary sensory cells (Mollicone et al. 1986a), but lacked these antigens on vascular endothelium and erythrocytes (Socha and Ruffie 1983).

Baboon. These animal express strong A and/or B antigens in exocrine secretions, in primary sensory cells (Mollicone et al. 1986a) and in vascular endothelium (Oriol et al. 1984). Three phenotypes A, B and AB have been described in their secretions, but the O phenotype has not been found as yet (Socha and Ruffie 1983). Expression of ABH tissue antigens in this species is intermediate between marmoset and man. They have ABH antigens on vascular endothelium as in man, but they lack ABH antigens on red cells as in lower mammals (Oriol et al. 1984).

Man. Higher antrophoid primates (Socha and Ruffie 1983) and man are the only species which express ABH antigens on red cells. Therefore the, so-called "major blood group antigens" whould better be defined as tissue antigens than as red cell antigens. In fact, they only appear on erythrocytes very late in evolution (Figure 1) (Oriol et al. 1986).

ABH	Digestive mucosae	Epidermis	Olfactory receptors	Posterior root ganglia	Vascular endothelium	Red cells
	+	+	+	−	−	−
	+	−	+	+	−	−
	+	+	+	+	−	−
	+	+	+	+	+	−
	+	+	+	+	+	+

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PHYLOGENY

ABH and related oligosaccharide antigenic determinants are widely distributed in nature.

An "A like" antigen was found in digestive secretions and on cortical granules of *Xaenopus leavis* eggs. The X or Le^x antigen (also known as SSEA1) was found in venom glands of poisonous snakes (Oriol et al. unpublished results). But most of these glycoconjugate structures in lower vertebrates or even in invertebrates have been detected only serologically, they have not been purified and their chemical structures are not well defined as yet.

Rat. Chemical analysis of glycolipids extracted from rat intestinal mucosa has enabled characterization of the terminal tetrasaccharide of the A antigen: αGalNAc(1→3)[αFuc(1→2)]βGal(1→3)βGlcNAc-R (Breimer et al. 1982). This antigen was present in the small intestine of certain rat strains, whereas other strains expressed only the H antigen αFuc(1→2)βGal(1→3/4)βGlcNAc-R, in the small intestine (Breimer et al. 1980). Unlike this, the A antigen was expressed in the colonic mucosa of all rat strains. Therefore, in the rat, A antigenic determinant expression is under polymorphic genetic control, restricted to the small intestinal mucosa alone. None of the strains of rat tested expressed A on the oral mucosa, but H and B were found in this area in all the rats examined (Reibel et al. 1984 and Reibel 1987). B and H antigens were also found in some primary sensory neurons of the rat (Mollicone et al. 1985a, Dodd et al. 1985), but no ABO related structures have been described so far in rodent erythrocytes or vascular endothelium.

Rabbit. Expression of the A antigen is polymorphic in the digestive mucosa of rabbits. There are A^+ rabbits, expressing the A antigen and A^- rabbits, which only have the precursor H antigen (Oriol et al. 1977). All rabbits express the B antigen in digestive mucosa irrespective of their A genotype, including homozygous A^+/A^+ rabbits. This suggests that A and B antigens do not behave like alleles in rabbits.

Some primary sensory cells of the rabbit also express A, B or H antigens. Erythrocytes of this species do not express the normal fucosylated ABH antigens, but they have an unfucosylated linear "B like" structure (Hanfland et al. 1981).

Pig. A and O pigs have been defined serologically using red cells, but the antigenic structures responsible for these serological reactions are probably circulating glycosphingolipids adsorbed onto red cells. Incubation of red cells from an O pig, in plasma of an A pig, transformed the O cells into A cells and viceversa, incubation of A red cells in O plasma resulted in a significant loss of A antigen (Oriol 1987). The digestive mucosa has large amounts of A antigen in A pigs and H antigen in O pigs.

Dog. Dogs have a very weak "A-like" antigen in red cells called Tr (Bowdler et al. 1971), which is probably adsorbed at the surface of erythrocytes as the "A-like" antigen of pig erythrocytes.

Four different phenotypes have been found in dog digestive mucosa A, AY, Y and X (Oriol et al. 1975). The Y antigen of the dog corresponds to the Y antigen of man, that is the difucosylated type-2 isomer of the Le^b antigen. The serologically defined X antigen of the dog (Zweibaum et al. 1974) has the structure of the H type-2 antigen of man (Mc Kibbin et al. 1981). Therefore, from a genetic point of vue, the dog has two distinct genetic

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Phylogeny and Ontogeny of Blood Group antigens. A study by Polychromatic Tissue Immunofluorescence of ABH and Related Antigens.

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CHEMISTRY AND GENETICS

ABH antigens can be built on six different precursor chains:

Type 1....βGal(1→3)βGlcNAc-R
Type 2....βGal(1→4)βGlcNAc-R
Type 3....βGal(1→3)αGalNAc-R
Type 4....βGal(1→3)βGalNAc-R
Type 5....βGal(1→3)βGal-R
Type 6....βGal(1→4)βGlc-R

Each of these chains can be transformed into the different monofucosylated and difucosylated A, B or H structures, by addition of terminal fucose, galactose or N-acetylgalactosamine residues (Oriol et al. 1986). Addition of these sugars is performed by the products of specific genes which work at three different levels (Table 1). At the first level, addition of fucose to subterminal N-acetylglucosamine, by products of the Lewis or X genes, produces the monofucosylated Le^a or X antigens on type 1 or type 2 chains respectively. At the second level, addition of another fucose, α(1→2) linked to terminal galactose of any of the six precursors, makes the monofucosylated H antigens. Addition of both fucose residues, at levels 1 and 2, makes the difucosylated Le^b or Y structures. Finally, at the 3rd level, addition of N-acetylgalactosamine or galactose in α(1→3) to any of the H structures makes the A or B antigenic determinants.

Level	Gene	Enzyme	Structure
3	ABO	A or B	**αGalNAc** or **αGal** (↘ ↙)
2	Hh; Sese	H or SE	**αFuc** → βGal ↓
1	Xx; Lele	X or Le	**αFuc** → βGlcNAc ↓ R

Table 1. Genes, enzymes and chemical structures resulting from interactions of the products of ***ABO, Hh, Sese, Lele*** and ***Xx*** genetic systems at three different levels of terminal oligosaccharides.

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V. Stains

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IDENTIFICATION IN BLOOD STAINS THROUGH DNA TYPING WITH C4 AND HLA-DR PROBES.

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A restriction fragment length polymorphism (RFLP) analysis using double digestion of DNA preparations with XbaI and BglII restriction enzymes is presented. In our panel of 46 unrelated individuals 37 different phenotypic patterns were recognized when using C4 and HLA-DR probes. The preliminary discriminative power value when employing both probes (consecutively or simultaneously) is 0.985. In 6 months old blood stains from 7 of the panel members the RFLP patterns were well preserved both in C4 and HLA-DR. The stains from all these individuals were identified when comparing stain DNA patterns with panel control patterns. Based on these laboratory experiments, it is concluded that DNA typing with such probes may become a powerful tool in future stain identification analysis.

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Figure 3.

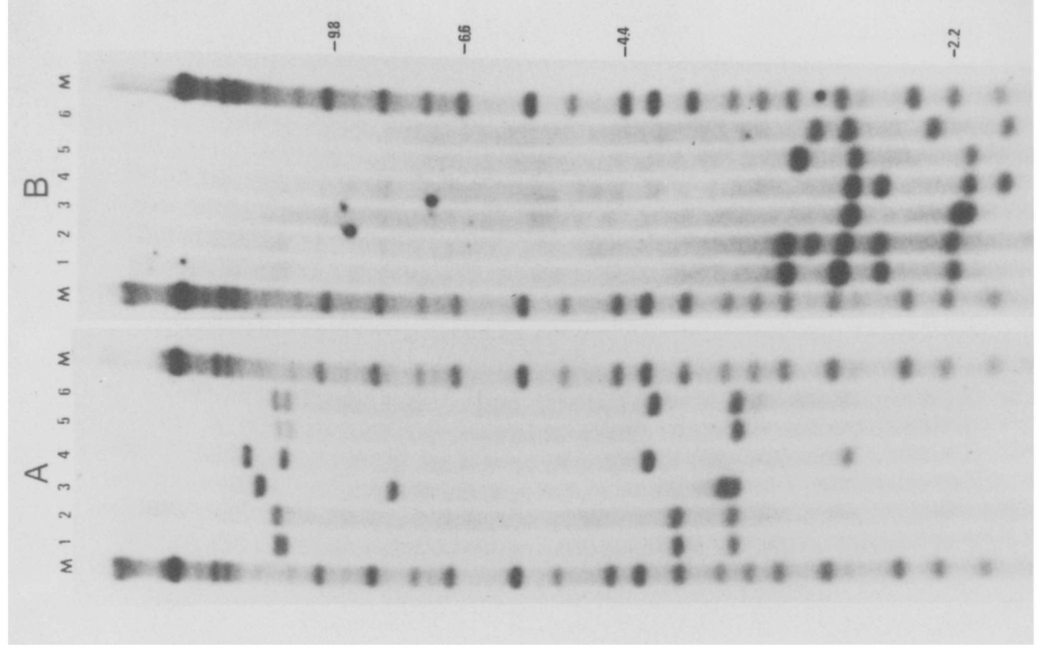

Figure 4.

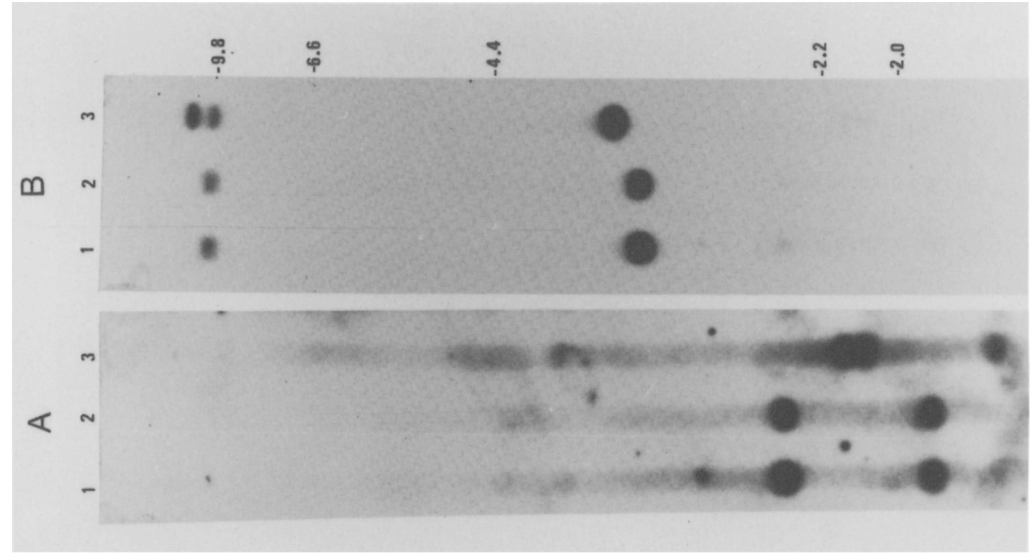

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Figure 1.

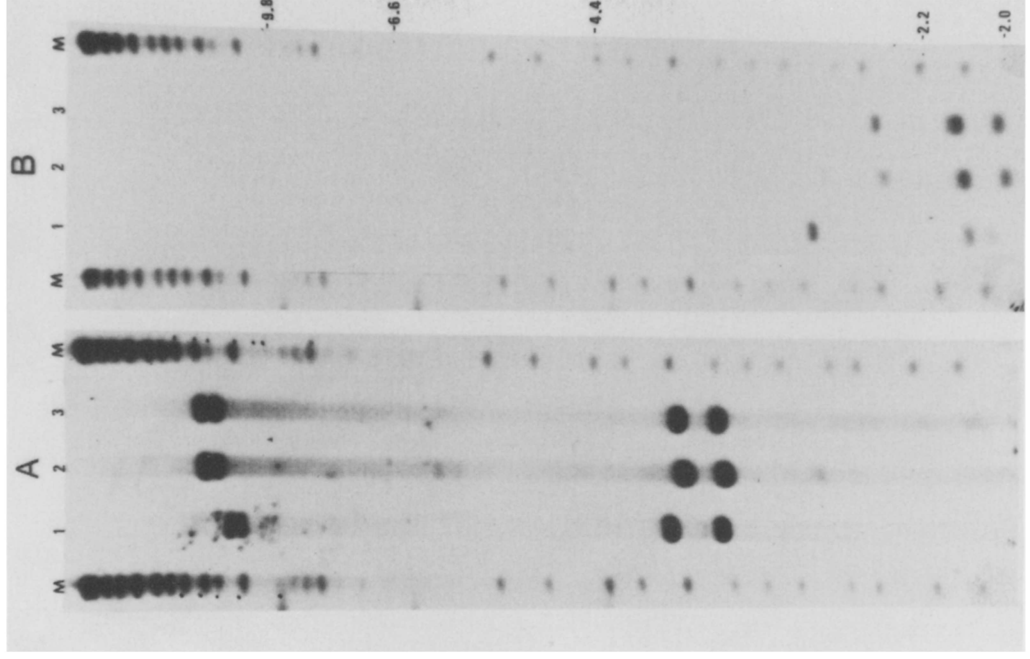

Figure 2.

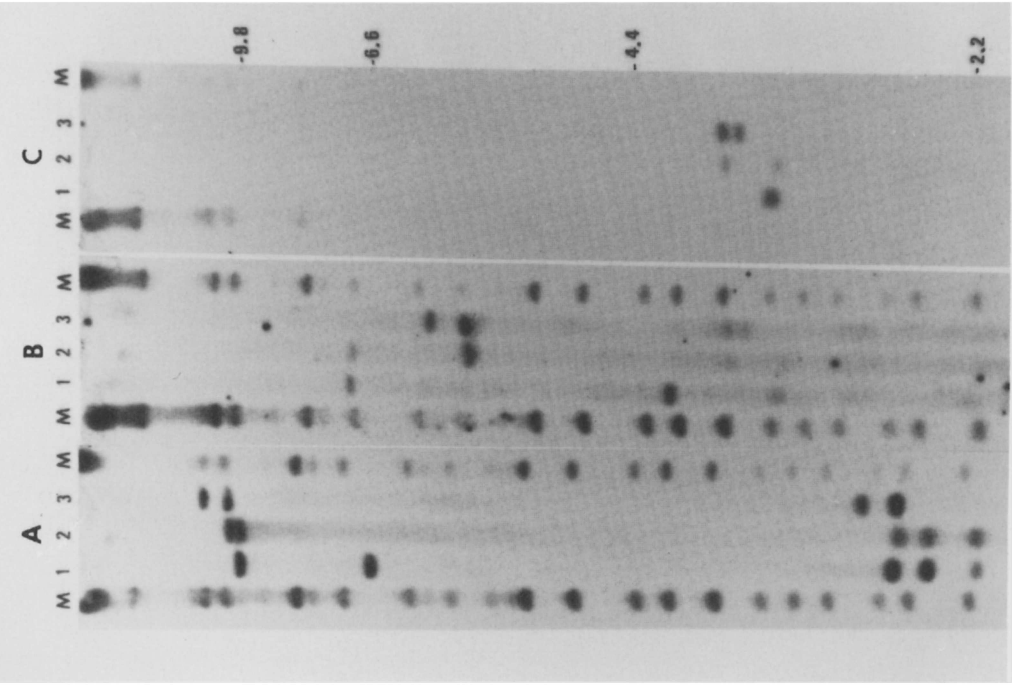

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Figure Legends.

Figure 1. DNA-Print pattern from a sexual assault case. Lane 1, DNA isolated from the victim. Lane 2, DNA isolated from the evidence, vaginal aspirate. Lane 3, DNA isolated from the suspect. M indicates size marker lanes and numbers along the side represent sizes in kilobases. (A). Results with pAC255 and pAC256. (B). Results with pAC225.

Figure 2. DNA-Print pattern from a homicide. Lanes 1 and 3, DNA isolated from putative mother and father respectively. Lane 2, DNA isolated from the evidence, brain tissue. (A). Results with probes pAC225 and pAC255. (B). Results with probe pAC061. (C). Results with probe pAC256.

Figure 3. DNA-Print pattern from a product of conception rape case. Lane 1, DNA from the rape victim. Lane 2, evidence (abortus material). Lanes 3-6 suspects 1 to 4 respectively. (A). Results with probes pAC255 and pAC256. (B). Results with probe pAC225.

Figure 4. DNA-Print results from blood stains. Lanes 1, DNA isolated from the homicide victim. Lane 2, DNA isolated from unknown blood stain. Lane 3, DNA isolated from the suspect. (A). Results with probe pAC225. (B). Results with probes pAC225 and pAC256.

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Table 1. DNA probes used for the analysis of Pst 1 digested DNA.

Probe	Specificity	Number of Alleles
pAC004	Y-specific	NP
pAC061	single locus	> 80
pAC225	single locus	>100
pAC255	single locus	> 50
pAC256	single locus	> 30
pAC101	human specific	NP
pAC267	bacteria specific	NP

NP: not polymorphic

Table 2. Summary of Forensic Cases.

CASE TYPE	NUMBER	SUCCESSFUL	NO HUMAN DNA	DEGRADED DNA
RAPE - ID	20	8	7	5
RAPE - PATERNITY	11	9	0	2
MATERNITY	3	2	0	1
PATERNITY - BODY ID	3	2	0	1
OTHER - STAINS	10	6	2	2

Table 3. Discrimination potential of 4 DNA probes used for identification.

	DNA probes				
	pAC061	pAC225	pAC255	pAC256	
Ethnic group					Total
Black	143	350	244	62	7.6×10^8
Caucasian	267	350	154	51	7.3×10^8

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the human DNA. This can lead to false estimates of the amount of DNA in the sample. In the other 60% of cases no significant number of sperms were observed. DNA purification from those samples yielded degraded DNA of bacterial origin or no DNA. The discrimination potential of a combination of four DNA probes that hybridize to hypervariable regions, in American Blacks and Caucasians, is shown in Table 3. The calculations were performed using the formula described by Fisher (7). The values represent the average number of people discriminated with each probe.

The following are examples of some of these forensic cases:

Case 1 - Sexual Assault. A vaginal aspirate was recovered from a rape victim. DNA was isolated from this sample as well as from peripheral blood of both victim and suspect. The amount of high molecular weight DNA recovered from the semen sample was approximately 25 ug. Results indicated that, using 3 probes, the DNA-Print pattern seen in the evidence (figure 1A,B lane 2) matched that of the suspect (lane 3) and it was different to that of the victim (lane 1). The frequency of the matching pattern in a Caucasian population was calculated as 1 in 12,000,000.

Case 2 - Homicide. A car was found abandoned and fragments of brain tissue were found in the grill of the car. The owner of the car was reported missing, but no body was found. DNA was isolated from the brain tissue (>100 ug of DNA) and also from the peripheral blood of the alleged parents of the victim. The DNA samples were hybridized to 4 probes and in each case the pattern of bands from the evidence (figure 2A,B,C lane 2) was consistent with having been derived from the alleged parents (lanes 1 and 3). Statistical analysis indicated that it was 160,000 times more likely that the DNA isolated from the brain tissue might have been from the offspring of the two parents than from a random individual.

Case 3 - Product of conception in a rape case. DNA was isolated from an abortus recovered from a rape victim. Four individuals were tested, with 3 probes, as possible biological fathers. The results excluded three of the suspects and included a fourth one (figure 3A,B lane 6) with a probability of paternity of 99.9%.

Case 4 - Homicide. A 2 cm blood stain on cloth was recovered from a murder scene. Blood samples were provided from the victim and a suspect. The DNA-Print patterns with 3 probes indicated that the unknown stain (figure 4A,B lane 2) matched the pattern of the victim (lane 1) and it differed from that of the suspect (lane 3). The frequency of the matching pattern in the Black population was 1 in 150,000,000.

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digitizing system as previously described (4).

DNA Probes and Data Analysis. The arbitrary DNA probes used for hybridization were derived from human genomic libraries. The following DNA probes recognize a single locus: pAC061, pAC225, pAC255, and pAC256. One DNA probe, pAC225 (derived from 29C1)(5) detects from one to six bands per person. The probe, pAC004, hybridizes to a 3.7 Kb Pst 1 Y-specific repeated DNA sequence and can be used to show the presence of male DNA. The probe pAC101 hybridizes to human specific repeated DNA fragments and can be used to confirm the human origin of the samples. The probe pAC267, derived from an E. coli ribosomal gene clone, kindly provided by Dr. Squires, Columbia University, was used to detect the presence of bacterial DNA. The properties of these probes are summarized in Table 1. The number of distinct alleles in the hypervariable loci varied from 30 to >100.

A database containing the frequency of the fragment sizes detected for each locus has been established using non-related American Black and Caucasian individuals. The allele frequency distribution of these polymorphic loci will be published elsewhere. For the calculations of identity, the allele frequency of a particular DNA fragment in the population was obtained using a mathematical expression incorporating band resolution and standard deviation (manuscript in preparation).

Results and Discussion

Following extraction of DNA from forensic samples, the amount and integrity of the DNA was determined by electrophoresis as described in Methods. If sufficient DNA was available for analysis, it was digested with Pst 1. About 0.1-0.5 micrograms of this digest was fractionated by electrophoresis on a gel and examined for completeness of digestion. The DNA was transferred to a nylon membrane and hybridized sequentially with the Y-specific probe (pAC004), the human specific probe (pAC101), and the bacterial specific probe (pAC267). The results of these hybridizations indicated whether the sample contained undegraded human male/female DNA, and the presence of contaminating bacterial DNA. The relative intensity of the hybridization signal with the human vs. bacterial probes gave a qualitative estimate of the amount of human specific DNA in the sample. Next, a diagnostic gel(s) was used to fractionate the DNA samples so as to maximize resolution in the 2-20 Kb DNA size range. Following transfer to a nylon membrane, the DNA from the diagnostic gel was hybridized to the ^{32}P-labelled probes that recognize hypervariable regions.

We have analyzed 20 cases of sexual assault where the evidence examined included semen stains on undergarments, vaginal aspirates, and/or vaginal swabs. Microscopic examination of the samples showed the presence of sperms in 40% of cases. In these cases sufficient human specific DNA was available for analysis and this was confirmed by the presence of specific hybridization signal with the probes recognizing the hypervariable loci. In 2 of the 8 successful rape cases (Table 2) we could detect the presence of some bacterial DNA contamination in the DNA isolated from evidence material. However, the amount of bacterial DNA was small and did not interfere with the interpretation of the results. Because of the non-sterile nature of the samples, naturally occurring bacteria can propagate and its DNA will co-purify with

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THE APPLICATION OF DNA-PRINT FOR IDENTIFICATION FROM FORENSIC BIOLOGICAL MATERIALS

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Abstract

DNA purified from more than 170 forensic samples was characterized by restriction fragment length polymorphism analysis. DNA isolated from dried blood stains, various tissues, and semen stains was digested with the restriction endonuclease Pst 1, size separated by agarose gel electrophoresis, transferred to nylon membrane and hybridized with DNA probes which recognize hypervariable regions in the human genome. The size of DNA fragments, reacting to these probes, was measured with a computer assisted digitizing system and a likelihood of identity calculated from databases containing the frequency of the alleles identified with each probe. The combined discrimination potential with 4 DNA probes that hybridize to hypervariable regions is greater than 10^8. The present report also summarizes some of our experience in the analysis of forensic biological evidence.

Introduction

Differences which occur at the DNA level can be visualized as restriction fragment length polymorphisms (RFLPs) and can be used as genetic markers for disease diagnosis (1) and identification (2,3). The use of a number of DNA probes which recognize single locus hypervariable regions allows the determination of a genotype (DNA-Print) characteristic for an individual (4). In cases where identification is necessary from forensic evidence, a DNA-Print can yield vital information as to biological origin. We present in this report results of the analysis of forensic samples and the power of this system to resolve issues of identity.

Materials and Methods

DNA Isolation. DNA was isolated from peripheral blood, dried blood stains, or semen stains as previously reported (2-4). Kidney, liver muscle, and brain tissues were minced and processed the same as peripheral blood. The quantity of DNA isolated was determined in a spectrophotometer at 260 nm and its integrity by electrophoresis in agarose gels. DNA (0.2-5.0 ug) was digested with the restriction endonuclease Pst 1 and size separated by electrophoresis on 0.9% agarose gels. Molecular weight standards composed of lambda bacteriophage and phi X174 DNAs cut with various restriction endonucleases were incorporated in each gel to allow size determination of the polymorphic DNA bands from the samples. After transfer to a nylon membrane, the blots were hybridized (2) to ^{32}P-labelled DNA probes (6) and exposed to X-ray film. If rehybridization of a filter to a different probe was necessary, the signal was removed by incubating 30 min. at 65°C in 50% formamide, 2XSSCP (2), 1% SDS. Filters could be reused at least 6 times without significant loss in sensitivity. The resulting autoradiographs were analyzed with a computer assisted

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or in absolute ethanol or waterfree aceton for longer periods without notable deterioration of high-molecular-weight DNA.

Tab.:
Summary of the quantitative and qualitative DNA yields of different human organ tissues

Tissue	Slope of post-mortem DNA-Degradation	Quality of DNA (highmolecular)	DNA-Yields µg DNA per mg tissue max	min
Brain Lymph Node Psoas Muscle	DNA / p.m.	+ + + + + + + + +	0.145 4.6 0.148	0.005 0.09 0.002
Spleen Kidney	p.m.	++ +	2.2 0.548	0.005 0.0002
Blood	p.m.	+ +++ (clots)	0.110	0.0005

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Quality of the recovered DNA

The overall quality of the extracted DNA was estimated as the amount of slow migrating high-molecular-weight DNA to faster moving "smearing" DNA of lower molecular weight for each organ. Increasing amounts of low molecular DNA in direct relation to postmortem age were obvious for all organ tissues but a few cases were especially marked by this DNA degradation independent of the postmortem period. Elevated room temperature as environmental factor and/or infectious disease before death were the common main causes for the advanced autolysis in these cases.
Stability of DNA in brain cortex was excellent for a period of 3 weeks postmortem. The quality of the DNA of lymph node extracts was at least as good as the one of brain. The stability of DNA of psoas muscle was also similar to brain and lymph node tissue. Some rare exceptions were probably a consequence of the differences of postmortem body cooling do to the quite different topographical localization of these organs in the human body. DNA degradation of splenic tissue was more rapid than in lymph nodes in some of the cases. A liquefying consistency of the splenic tissue was typical in these cases and autolysis was usually also a prominent feature. In our experience, high-molecular-weight DNA was found in only small amounts in splenic tissue after 4-5 days postmortem.
Degradation of DNA of kidney tissue was almost total after 5 days in the 11 cases examined.

Storage of tissue specimen in absolute ethanol or waterfree aceton preserved high-molecular-weight DNA. After thorough washing of the tissue samples in sodium chloride, no influence of the fixatives on the quality of DNA was observed. However, preservation of the tissues in a solution of 4%-formalin resulted in negative yields of DNA.

DNA-FINGERPRINTING ON POSTMORTEM TISSUE SPECIMEN

DNA-fingerprinting using the minisatellite probe 15.1 was performed on all the samples. Basically identical banding patterns were found for different tissues of the same individual. Gradual disappearance - fainting to complete loss - of the long fragments in the 15-20 kb range was sometimes noticed. This alteration could not strictly be correlated to the postmortem age of the samples but appeared to be a consequence of the degree of autolysis. Organs commonly showing a higher degree of autolysis like liver, spleen and kidney sometimes showed this fainting of the long fragments after only a few hours postmortem. Nonspecific fragmentation of DNA seems to primarily affect the longer fragments. But since the resulting fragments are all of shorter but never identical length, no extrabands are to be expected on the fingerprints and therefore false exclusions do not occur. However, the evidentiary value of the fingerprint is lower. Cases with complete loss of high-molecular-weight DNA never showed recognizable banding-patterns after hybridisation. An amount of 1 to 60 mg of crude tissue was usually sufficient to produce a DNA-fingerprint.

CONCLUSIONS

We found that in principal high-molecular-weight DNA could be recovered postmortem in large quantities from various human organ tissues as well as from blood, but not all organs were equally well suitable. Excellent DNA stability was found in brain cortex, lymph nodes and psoas muscle over a period of three weeks postmortem. Degradation was relatively slow. Spleen, kidney and thyroid gland showed good DNA stability up to 5 days postmortem but after longer periods, very rapid degradation was usually observed. Yields of DNA of blood were very variable do to inhomogeneous samples. Blood clots gave very good results. Generally, the degradation of highmolecular DNA correlated directly to the length of the postmortem period (Tab.). However in some cases; DNA degradation was already prominent after a very short period and all organs were equally touched. Case histories showed that high environmental temperature at the place of death and/or infectious disease prior to death were the main factors for the occurrence of rapid autolysis.Tissue specimen can be stored frozen at -20°

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EDTA, 100 mM NaCl (pH 8) and 40 mM DTT with a mechanical homogenizer of Popper. After adding 2% SDS and 40μg/ml Proteinase K the homogenized tissue was incubated at 37°C overnight . DNA-extraction was done three times for 10 minutes in equal volumes of a 1:1 mixture of phenol and methylene chloride and in 1 volume of methylene chloride for again 10 minutes. DNA was precipitated by adding 1/10 volume of 3 M Na-acetate and 2.5 volumes of absolute ethanol followed by freezing at -80°C for a least 2 hours. Recovery of low quantities of DNA necessitated centrifugation for 10 to 15 minutes at 13000 rpm. After vacuum drying for 20 minutes, the pellets were dissolved in appropriate volumes—usually in 20 to 400 μl— of TE-buffer of pH 7.6 . The yields of DNA were estimated semi-quantitatively on testgels for each specimen by direct comparison with ethidium-bromide stained λ-DNA-markers of 50, 100, 200 and 500 ng DNA. The mean value of these 4 estimates per organ and case and its standard deviation were calculated and expressed in microgram of recovered DNA per milligram of crude tissue. Theses values were plotted against the postmortem period for all organ tissues. Approximately 0.2 to 1 μg of DNA was sheared with 20 units of the restriction enzyme Hinf I at 37°C overnight, electrophoresed in a 22 cm 1.2 % agarose gel (SIGMA II A 8677) for 48-65 hrs and Southern blotted onto nitrocellulose filters. The ^{32}P-labelled probe was prepared from the human minisatellite M13 recombinant 33.15 after insert preparation of replicative forms by double digestion with EcoRI and HindIII and random oligolabling according to Feinberg and Vogelstein using the commercially available Multiprime System of Amersham (RPN.1601). Southern blots were hybridized overnight at 42°C in the presence of 50μg/ml denatured salm sperm, 0.1% SDS, 6% polyethylene glycol 6000, 45 % formamid , 10x Denhardt´s and 1xSSC. After washing the filters with 1xSSC for 10 minutes at room temperature and for 1 hr at 65°C, they were autoradiographed for 1 to 5 days at -80°C using 2 intensifying screens.

RESULTS

Quantitative Yields of DNA

Brain Cortex: The meanvalues of DNA of the 4 extractions varied considerably and ranged from 0.004 μg to 0.14 μg per milligram of crude tissue in 24 cases. A steady exponential decline of the yields of DNA in relation to the postmortem age was observed. The correlation coefficient of these two variables was acceptable (R=0.62).
Lymphatic tissue: Do to the abundance of cell nuclei in lymphatic tissue, DNA yields per milligram of tissue were about 10-15 times greater than those of brain cortex (0.09 to 4.6 μg of highmolecular DNA per mg crude tissue). In 17 cases of cervical lymph nodes, a steady decline of the DNA yields in relation to the postmortem age was again observed. However, the correlation coefficient of these two variables was lower (R=0.55).
Psoas Muscle: In 24 cases, the meanvalues of the yields of 4 DNA extractions of psoas muscle were of the same order of magnitude as the ones of brain cortex and varied between 0.003 to 0.100μg.
Blood: Yields of highmolecular DNA of postmortem blood samples showed a considerable variation and ranged between 0.0005 to 0.113 μg per microliter blood in 23 cases investigated. A direct correlation of the yields to postmortem age was not found. It was shown that blood samples drawn from vessels were not homogeneous. Presence of small clots in the sample increased the DNA yields considerably and was found to be responsible for the great quantitative variations of DNA yields. DNA extractions of blood clots that are often present in decayed bodies gave in fact the best results.
Spleen: DNA yields of splenic tissue per milligram tissue were about 2 times lower than those of lymph node in 18 cases and varied between 0 to 2.2 μg per mg crude tissue. Decrease of highmolecular DNA was exponential with a good correlation to postmortem age (R=0.84).
Kidney: Tissue of renal cortex was extracted in 13 cases. DNA yields ranged between 0 and 0.54 μg and dropped exponentially with a good correlation to postmortem age (R=0.84) with one exception.
Liver: Liver tissue always showed a high proportion of degraded DNA independant of the postmortem period and therefore yields could not properly be estimated.

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DNA: POSTMORTEM STABILITY IN VARIOUS HUMAN ORGAN TISSUES

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INTRODUCTION

The detection of DNA polymorphisms considerably expanded the means of discrimination of individuals based on small tissue samples. So-called "DNA-fingerprints", a banding pattern generated by hypervariable minisatellites are unique for every individual, except for monozygotic twins (Jeffreys et al., 1985a, 1985b). The bands are inherited according to the law of Mendel, hence familial relationship is reconstructible by comparing fingerprints of the parents or close relatives with the one of the unknown (Jeffreys, 1986). Questions of identity in forensic cases often concern remains of humans who have been dead for a shorter or longer time. Since high-molecular-weight DNA is a prerequisite to obtain reliable RFLP-patterns, it is important to investigate the postmortem stability of DNA under various conditions. Reports of Pääbo in 1985 on isolation of DNA from mummies of 2000 years of age amplified the enthusiasm to "raise (genetically) the dead and buried" as Jeffreys expressed it 1984. However, only short length DNA-fragments or none at all were recoverable so far (Huges et al., 1986). Postmortem decay of human bodies is an extremely complex and not yet fully understood process which consists of an aerobic and bacterial decomposition of organic material. It starts with autolysis followed by putrefaction. Autolysis is a nonbacterial autodigestion of tissue by enzymes liberated from the lysosomes whereas putrefaction is an anaerobic bacterial decomposition of proteins which is often accompanied by production of gas. However, the two processes usually cannot be clearly separated and both show a maximum of activity at temperatures between 34-40 ° Centigrade. In autolysis, loss of enzyme regulation and lactic acidosis enhance the activity of some enzymes, e.g of the hydrolases, whereas other's activity is reduced. Surprisingly, most of these enzymes are remarkably resistant to autolysis itself. But they are usually rapidly destroyed by bacteria. DNA in dead cells itself is degraded by nucleases which belong to the large enzyme group of the "hydrolases". Two major groups exist: endonucleases and exonucleases. Endonuclease decompose the DNA from inside the DNA-strand by randomly shearing it into smaller fragments whereas exonucleases cut single nucleotides one after another from one of the terminal ends, thus gradually shortening the fragment. We have investigated the yields of undegraded DNA extracted of human tissue specimens. We examined tissue of brain, lymph nodes, liver, spleen, psoas muscle, kidneys and blood of human bodies of various postmortem age. Nonspecific degradation of highmolecular DNA by random fragmentation could lead to the nonpredicable appearance of extra-bands in DNA-fingerprints.To study possible postmortem changes of the banding patterns, DNA-fingerprinting was done on all the samples . Finally, preserving agents other than freezing for the storage of tissue specimen until DNA extraction were tested in a few cases.

MATERIAL AND METHODS

Tissue specimen of brain cortex, lymphatic node, liver, spleen, psoas muscle, kidney and blood of bodies of known postmortem age were collected from autopsy-cases of the University Institute of Forensic Medicine of Zurich. A maximum of 29 cases were examined. The bodies were "naturally" aged cases recovered in the county of Zurich and were not exposed to artificial ageing. The postmortem periods ranged from 0.2 to 19 days. The causes of death were heterogeneous. Since the amount of nuclear mass varies considerably in different organ tissues from very high in lymphatic tissue to low quantities in skeletal muscle, four blocks of 50 to 400 mg of tissue were separately collected, weighted and kept at 4° C if handled the same day or frozen at -20°C until homogenizing in a solution of 1 ml of 10 mM TRIS-HCl (pH 7.6), 10 mM

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like identification of bodies or parts of a body, provided some antemortem material can be used for comparison.

However, we do not expect a general introduction of this technique into paternity testing in Germany in the near future. As has been stated already, "yes" or "no" decisions can be made in the majority of cases using conventional typing. In most cases, the true father is alleged. This results in non-exclusion in all polymorphic systems studied. If the man is not excluded, German legislation strictly demands a calculation of the probability for fatherhood based on the Essen-Möller approach. For a system being included in the blood group expertise, the following prerequisites must be fulfilled:

1) estimation of allelic frequencies in the German population,
2) proof of dominant inheritance by segregation analysis,
3) negligible occurrence of mutations or other genetic events.

These conditions are not yet given in the analysis of minisatellite DNA. Estimates of mutation rates are as high as 1/240 (Jeffreys et al., 1985); haplotype frequencies cannot be calculated due to the lack of information about chromosomal localization, allelic relationship, and independence as basis of combinational probabilities. Thus, neither the combined exclusion chance nor the total probability in favor of the fatherhood of a given man can yet be addressed to the Court.

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mother of the child. Vice versa, all informations should be accounted for which are not derived from the grandmother, but from the grandfather (PV2), which are again not present in the mother, but in the child, and may be missing in the brother (PV1) of the mother. By careful inspection, only fragments of the first category can be ascertained: fragments of 12.5, 5.8, and 3.5 kb do occur in the grandmother, but not in the grandfather (PV2), are missing in the mother, but are present in the child and the brother (PV1) of the mother. No fragment of the second category was found.

By definition, all restriction fragments segregate in a dominant fashion. Thus, one of the parents must possess a given fragment observed in the child's DNA. On the basis of these findings it could be concluded that the grandfather cannot be the true father of the child K.

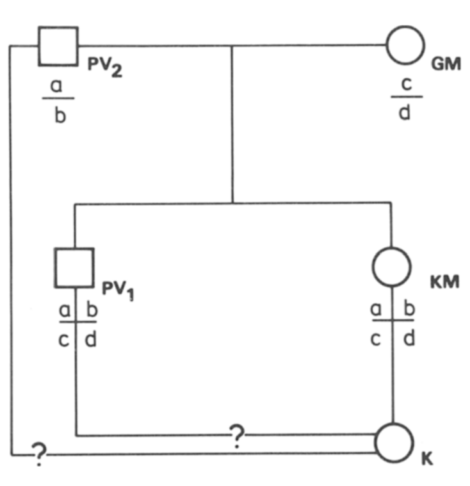

Fig. 1. Pedigree of family H.

Fig. 2. Autoradiogram of a Southern blot of family H after digestion of familial DNA with Hinf I and hybridization with M13.

DISCUSSION

The detection of highly polymorphic mini-satellite DNA by the commercially available phage M13 provides a new tool for the solution of incest and other problematic cases, which cannot be solved by conventional blood group expertise, even after the inclusion of the HLA system. The analysis of repetitive intron DNA is also well suited to detect a single individual out of a number of possible offenders on the basis of trace evidence (Gill et al., 1985), and in a number of further forensic applications

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is omitted as competitor for unspecific binding during hybridization. Therefore, we applied this procedure with some modifications to solve the incest case.

MATERIAL AND METHODS

Genomic DNA was purified from peripheral blood cells according to standard procedures. Individual DNA samples of 10 µg each were digested with 50 U of restriction endonuclease Hinf I (Pharmacia) in a total volume of 300 ul according to manufacturer's instructions. After digestion, the samples were extracted once with an equal volume of 1:1 phenol/chloroform, precipitated in ethanol and resuspended in 10 mM Tris, 1 mM EDTA, pH 6.8. Agarose gel electrophoresis was carried out in a 0.8 % gel using a 26 mM Tris, 13 mM Sodiumacetate, 1.3 mM EDTA, pH 7.7 electrophoresis buffer. The gels (20 x 20 cm) were run at a constant voltage of 30 V for 20 hours. After completion, the gels were treated with 250 mM HCl for 15 min., then twice with 0.5 M NaOH, 1.5 M NaCl for 30 min., and finally twice with 0.5 M Tris, 1.5 M NaCl pH 7.4 for 30 min.. The gels were blotted onto nitrocellulose filters (Schleicher & Schüll, BA 85) overnight in 16x SSC.

Prehybridization was carried out for 4 hours in a hybridization mixture as described by Johnson et al. (1984). The blots were hybridized with the M13 probe in the same solution for 15 hours. As hybridization probe, the double-stranded form of M13mp18 (Boehringer, Mannheim) was labeled by nick translation with ^{32}P-dCTP to a specific activity of 2 x 10^8 cpm/ug, and used at a concentration of 10^6 cpm/ml hybridization mixture. After hybridization, the filters were washed once for 15 min. in 2x SSC, 0.1 % SDS at room temperature, twice for 15 min. in 2x SSC, 0.1 % SDS at 50°C, four times for 30 min. in 2x SSC, 0.1 % SDS at 65°C, once for 30 min. in 1x SSC, 0.1 % SDS at 65°C, and once for 30 min. in 1x SSC at 65°C. The filters were exposed to Fuji RX film at -70°C with intensifying screens.

RESULTS

We used the procedure described above in a number of experiments with related and unrelated individuals, including monozygotic twins (Rittner et al., unpublished). We confirm that unrelated individuals rarely share given kb fragments, whereas monozygotic twins do not differ in their RFLP patterns.

The resulting DNA patterns of family H. are shown in Fig. 2. As could be expected for intrafamilial analysis, a number of fragments can be detected in all individuals, mainly in the size range of 20 to 25 kb. Some bands show segregation from the grandfather and the grandmother, respectively, to the offspring. To differentiate both putative fathers, only those informations are suited which are present in the grandmother and not in the grandfather (PV2), and do not segregate to their daughter, the

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APPLICATION OF DNA POLYMORPHISMS IN PATERNITY TESTING IN GERMANY: SOLUTION OF AN INCEST CASE USING BACTERIOPHAGE M13 HYBRIDIZATION WITH HYPERVARIABLE MINISATELLITE DNA

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INTRODUCTION

More than 25 blood, serum, and enzyme polymorphisms have been introduced into paternity testing in Germany in recent years (Rittner, 1975). If a "no" decision is defined by exclusion, and a "yes" decision requires a probability of 99.73 % or more, more than 90 % of court cases can be solved in this respect. A few cases not being clarified by a standard expertise include:

1) Cases with more than one alleged man if the men and/or the mother and the men are related.
2) Some cases where the putative father is deceased, and neither the parents nor the legitimate offspring are available for the study.
3) Cases where possible exclusion in a given polymorphic system interferes with an overall evidence in favor of paternity of the alleged man. The "exclusion" could then be traced back to a mutation.

Even some of such cases can sometimes be led to a conclusion, particularly if the HLA antigens can be determined. We have shown recently that a sixteen weeks old fetus could reliably be typed for HLA, thereby providing the paternity of the father of the child's mother with a confidence of 99.936 % probability (Rittner et al., 1986).

In another case (H./H., see Fig. 1) even after the inclusion of more than 30 blood, serum, and enzyme polymorphisms, including HLA-A,B,C, we were unable to distinguish PV_1 - the brother of the mother - from PV_2 - the father of the mother - as putative fathers of her child.

To obtain further information, we applied the DNA technology based on the detection of so-called hypervariable mini-satellite DNA, more widely known as "DNA fingerprinting" (Jeffreys et al., 1985). Such non-coding, highly repetitive DNA "core" sequences are scattered over the genome with unknown chromosomal localization. Therefore, the allelic relationships of their restriction fragments could not yet be elucidated. The repetitive intron sequences are phylogenetically highly conserved as simple repeats. So it was not surprising that similar sequences could be detected with oligonucleotide probes specific for "GAT/CA" repeats (Ali et al., 1986).

In 1987, Vassart et al. reported that the single-stranded bacteriophage M13, generally used as cloning vector for DNA sequencing, also detects a distinct set of hypervariable minisatellites in human and animal DNA, provided that herring sperm DNA

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HLA TYPING ON SINGLE HUMAN HAIRS: DNA PROBES TO ENZYMATICALLY AMPLIFIED GENES.

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A single human hair does not contain enough DNA to allow detection of DNA polymorphisms routinely (1). However, a new technique (2) is available that can greatly amplify, in-vitro, short regions of DNA and can detect as little as a few copies of a gene. By using this technique, called the Polymerase Chain Reaction (PCR), to first amplify DNA sequences present in hair, DNA polymorphisms can be easily detected both in single hairs that have been plucked and in single hairs that have fallen out.

In particular, a region of the class II HLA gene, DQ alpha, has been amplified by PCR from single hairs and the particular alleles of that gene present have been detected by hybridization to allele-specific, oligonucleotide probes (3). This has been done in a "dot-blot" format using either radioactive or non-radioactive probes. The "HLA typings" obtained have been verified by independent typing of the same individual's blood. PCR amplification can even be carried out on crude hair preparations without purifying DNA. These techniques promise simple, sensitive and rapid means of determining genetic variation between individuals.

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2. Saiki, R.K., et al., Science 230: 1350-1354 (1985)
3. Saiki, R.K., et al., Nature 324: 163-166 (1986)

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parentship, whereas all other probes show segregation of parental bands. Since recombination frequency has been calculated to be around 1o% in the 5´insulin gene region the reported RFLP can not be calculated in the segregation analysis.

Table 1. Problems in view of practical application of "DNA"-tests in paternity testing

Sample:	quantity	5-1o ml of uncoagulated blood
	quality	DNA should best be prepared from fresh leucocyte sample
		high molecular weight DNA (> 4okb)
Costs:	equipment costs around 15.ooo - 2o.ooo US$	
	fixed costs 1oo.ooo US$	
	cost per analysis (1 individual, 1 enzyme, 3 probes) 15o US$	
Time:	DNA preparation - 2days	
	RE-digest - 1-2 days	
	Electrophoreis - 3 days	
	Southern transfer - overnight	
	Hybridization - 2-3 days	
	Autoradiogram - 1-3 days	
Skill:	highly standardized lab	
	sterile work, if possible	

DNA technology is at present clearly second to the classical serological methods concerning practicability and reproducibility, since DNA analysis requires more hands-on time, and is considerably more expensive (Tbl.1). The goals of the molecular approaches to the human genome are both to obtain an exhaustive description of the human genome, and to be able to rapidly and inexpensively compare the genomes of many individuals, which will then provide new data on population genetics and will be the basis for parentship analysis. But at present, the number of persons screened as well as the number of familiy data are much to small in order to allow the calculation of recombinatorial events.

Acknowledgements.
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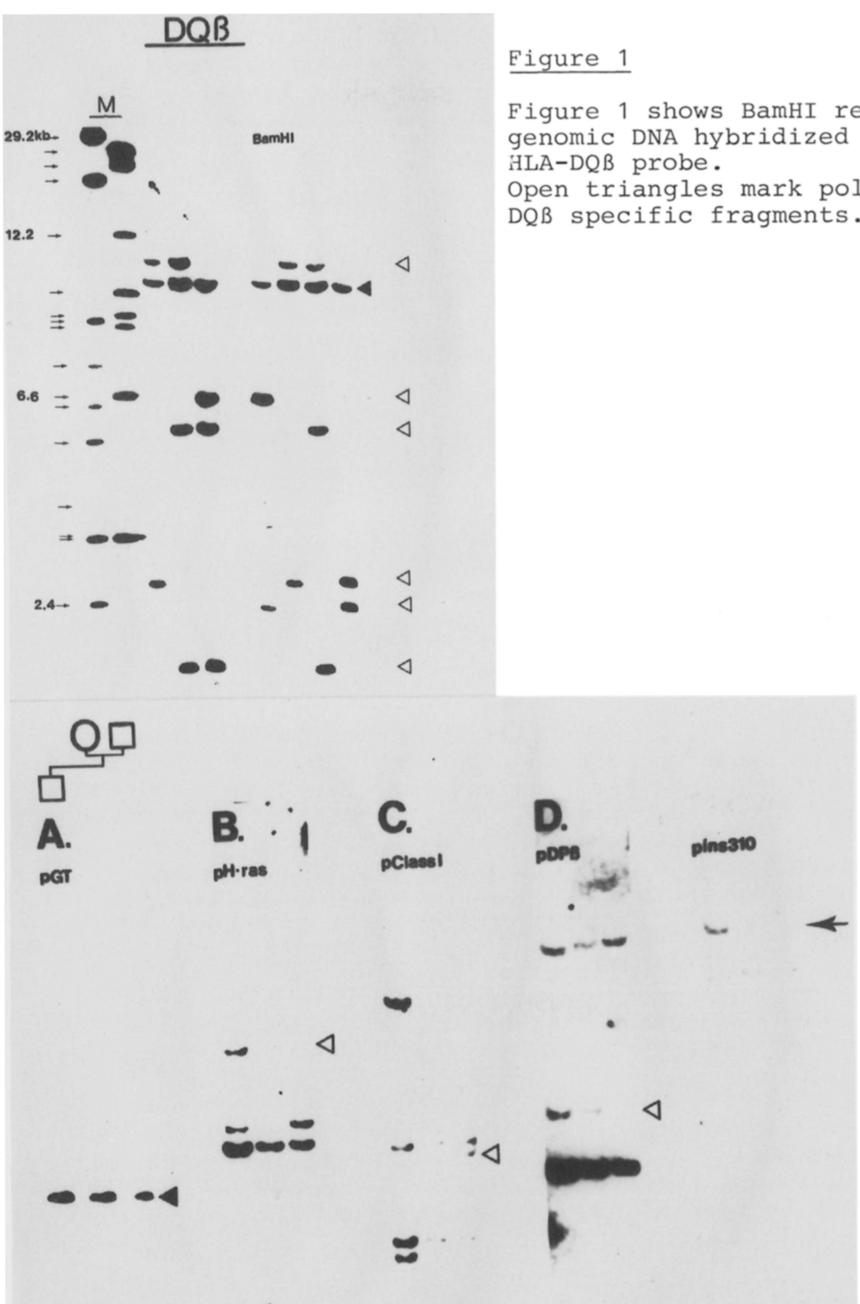

Figure 1

Figure 1 shows BamHI restricted genomic DNA hybridized with a HLA-DQß probe.
Open triangles mark polymorphic DQß specific fragments.

Figure 2. Figure 2 shows segregation of various HLA and chromosome 11 specific restriction fragments. In part A a non-polymorphic probe was used in order to proof complete digestion.

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salt conditions. Restriction endonucleases are added at three steps with a final concentration of about 5-1oU/µg of DNA. Digests are performed overnight. With TaqI, a final concentration of 5U/µg of DNA is used. Incubation lasts for 6-8 hrs only. Most important, TaqI is insensitive to inpurities in a DNA preparation and yields an extensive series of RFLP´s within human sequences.

Completeness of digestion:

Evaluation that the restriction endonucleases have cut all the possible recognition sites is the most crucial point in Southern technique when applied to segregation analysis.
The evaluation of complete digestion depends on three complementary approaches. A) The high molecular weight DNA is broken into smaller pieces by the restriction enzymes and gives at control electrophoresis a homogenous smear with distinct bands which correspond to repetitive sequences. If low amounts of DNA, or unspecifically degraded DNA is loaded,repetitive sequences may no longer be visible.B) An aliquot of the digest-mixture is mixed with 1-2 µg of phage lambda DNA. Phage lambda DNA then gives a distinct pattern with a given enzyme which superimposes on the homogenous smear of genomic DNA. Note that phage lambda DNA can be preferentially digested while genomic DNA is still not restricted completely. C) After a complete digestion a Southern transfer is performed. This blot is hybridized with a non-polymorphic probe (Fig.2a). Only invariant bands should now be visible on the autoradiogram. Also efficacy of transfer of the DNA to the filter can be calculated with this approach. After having loaded the same amount of DNA, transfer is not identical for all the DNA´s loaded. This fact renders the calculation of gene dosage a rather difficult task. D) Starting with a new sample, a second restriction digest should be prepared. The same pattern should be observed.

Southern transfer:

A great variety of transfer protocols do exist. Most convenient is the transfer on a nylon-membrane, which allows a series of up to 2o hybridizations.Transfer buffer should be either SSC or SSPE, whereas alkaline transfer gives good results for high molecular weight DNA only.

RESULTS AND DISCUSSION

By use of probes corresponding to the human MHC as well as the hypervariable regions of the human insulin and H-ras gene locus, a variety of informative restriction fragment length polymorphisms can be defined (Fig.1 &2).
Fig.1 gives an example of an RFLP which resides within the DQß locus. The gel allows a resolution of bands from 24kb down to 2kb. Fig.2 gives an example for parentship analysis. In the first part (A), hybridization is given with a non-polymorphic probe. Part B to E shows the successive hybridizations with probes corresponding to the MHC and the chromosome 11. Note that the probe corresponding to the 5´-HVR of the human insulin gene -ins310- gives an exclusion for

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Segregation analysis and determination of parentship by use of RFLP´s corresponding to genes of the MHC on chromosome 6 and the short arm of chromosome 11

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INTRODUCTION

Due to molecular cloning of a great variety of human DNA sequences the human genome is now accessible to a direct analysis at the genomic level. By use of restriction analysis, polymorphisms can be described with a panel of restriction endonucleases by use of Southern technique.
For segregation analysis and determination of parentship we have chosen gene probes corresponding to the human MHC gene region and the human insulin locus and the H-ras proto-oncgene, both of which reside on chromosome 11. The MHC locus is known to be highly polymorphic at the product level. This is not the case for the ras and the insulin-gene locus. But this sequences contain so-called hypervariable segments either 5´or3´ to the corresponding coding sequences and permit therefore a series of useful RFLP´s.
Although DNA restriction analysis permits a segregation and thus also parentship analysis at the genomic level, series limitations of this technique have to be overcome before a routine use, most probably as an adjunct to conventional serotyping, is in sight.

MATERIALS AND METHODS

We will focus in this part on the quality standard when performing RFLP analysis.
DNA isolation:
5-1o ml of EDTA blood yields enough DNA (about 25 to 5oug) for a series of restriction analyses. Blood should not be anticoagulated with heparin since this basic protein inhibits several enzymes even after several purifications. For practicability EDTA blood can be shipped on dry ice and stored about 4 weeks at -2o°C before DNA is extracted. For a restriction analysis high molecular weight DNA (size > 4okb) should be preferred. Unspecifically degraded or mechanically sheared material gives high background or even loss of high molecular bands in Southern blots. DNA concentration is usually determined by two ways,a) DNA spectrofotometry and b) gel electrophoresis with phage lambda DNA as a reference.
Restriction enzyme digests:
Restriction analysis can be achieved most conveniently with a set of three buffers, low,medium and high salt. Spermidine at a final concentration of 1 mM can be added immediatly before the digest. It will improve digestability for medium and high salt conditions, whereas inhibition was observed under low

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DISCUSSION

Using a full length cDNA encoding the enzyme glyceraldehyde 3-phosphate dehydrogenase we have identified an RFLP in the human genome generated with the restriction enzyme Taq 1. This RFLP represents a simple two allele system with restriction fragments of either 3.9 kb or 2.7 kb present in Taq 1 digests of chromosomal DNA. The frequencies of the two alleles is 0.745 for the 3.9 kb allele or 0.255 for the 2.7 kb allele in over 100 random samples analyzed. The RFLP is passed in Mendelian fashion as an autosomal trait and analysis of somatic cell hybrids has localized the RFLP to chromsome 12. The functional gene encoding the G3PD enzyme has been mapped to chromosome 12 using somatic cell hybridization techniques (Bruns and Gerald 1976).

Multiple gene sequences encoding G3PD have been found in the human genome (Benham et al 1984, Hanauer and Mandel 1984, Tso et al 1985). The one presumed functional gene has been localized to chromsome 12 (Bruns and Gerald 1976) however, other sequences have been found on the X chromosome (Benham et al 1984, Hanauer and Mandel 1984). The sequence(s) on the X chromosome were found to exhibit characteristics of a non-functional pseudogene and thus remain silent. The Taq 1/pG3PD RFLP must be either located within an intron region in the functional G3PD gene or be located within a region closely flanking the gene that is not present in G3PD gene sequences located on other chromosomes since the RFLP behaves as a single genetic trait.

We are presently searching for other restriction enzymes that generate RFLPs detectable with the pG3PD probe. Since a large number of G3PD related gene sequences exist within the genome, it may be possible to identify RFLP on other chromosomes that would segragate independently of the Taq 1 RFLP and make the pG3PD probe a more informative tool in our paternity testing program.

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Aliquots of chromosomal DNA were digested wiith Taq 1 electrophoresed in agarose, blotted onto a nylon membrane and hybridized to nick translated insert from the pG3PD plasmid as described in Materials and Methods.

The gene frequencies of the different alleles are 0.745 for the 3.9 kb allele and 0.245 for the 2.7 kb allele based on the analysis of over 100 random samples of chromosomal DNA. These gene frequencies also hold for a more limited sampling of chromosomal DNA obtained from blacks. The Taq 1/pG3PD RFLP is passed in families as an autosomal marker in a Mendelian fashion (Fig. 2).

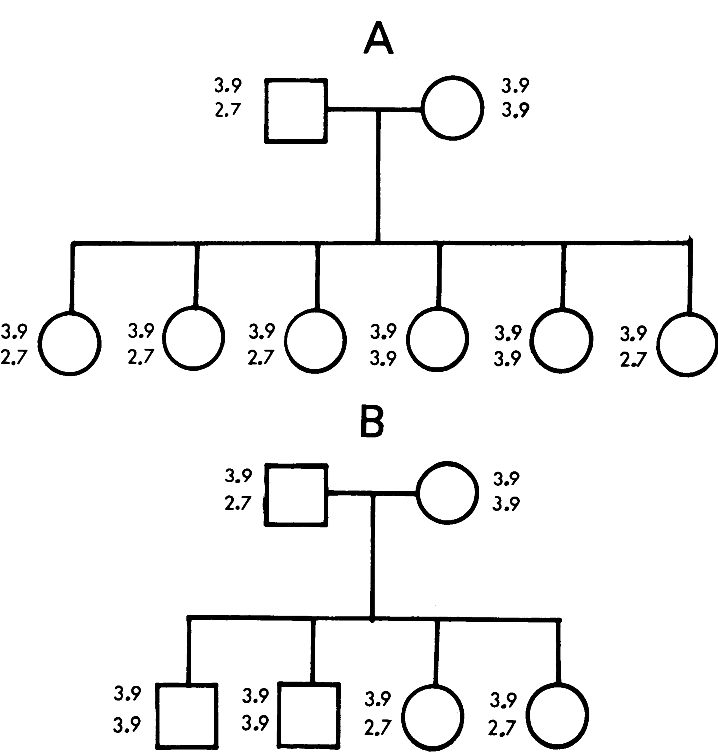

Figure 2 -- Mendelian inheritance of the pG3PD/Taq 1 RFLP in two families.

RFLP analysis was performed as in Figure 1 using chromosomal DNA from family members. Circles represent females and squares males in the figure.

The chromosomal location of pG3PD/Taq 1 RFLP was determined by analyzing chromosomal DNA from human-mouse somatic cell hybrids (kindly provided by Dr. F. Ruddle, Yale University) containing a limited number of human chromosomes. Results indicate that the RFLP is located on chromosome 12 (not shown).

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RESULTS

Numerous restriction fragments present in Taq 1 digests of chromosomal DNA hybridized to the pG3PD probe (Fig. 1) in keeping with the large number of G3PD sequences present in the human genome (Benham et al 1984, Hanauer and Mandel 1984, Tso et al 1985). The majority of these fragments were invariant in digests of random samples of chromosomal DNA. However, a Taq 1 polymorphism is also present in the digests representing a simple two allele system with fragments migrating with apparent sizes of either 3.9 kb or 2.7 kb (Fig. 1). The intensity of hybridization of the probe to the polymorphic fragments varies depending upon the genotype of the particular individual. Thus, in individuals homozygous for one of the alleles, the polymorphic band is roughly twice the hybridization intensity of the same band in a heterozygous individual (Fig. 1).

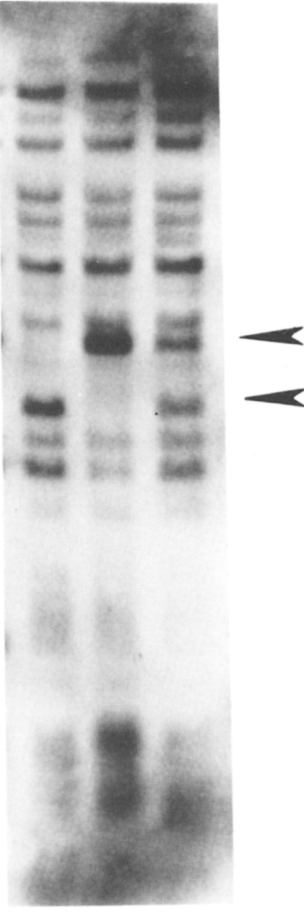

Figure 1 -- Hybridization of the pG3PD cDNA to a Southern blot of chromosomal DNA digested with Taq 1.

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We have been involved in studies aimed at evaluating the feasibility of applying RFLP mapping to routine paternity testing. As part of our effort, we have been systematically testing a variety of cloned cDNA fragments for their ability to detect RFLPs in samples of chromosomal DNA obtained from random blood donors. We recently reported the isolation of a cloned cDNA encoding the enzyme glyceraldehyde 3-phosphate dehydrogenase (pG3PD) (Allen et al 1987). We have used this cloned probe to determine if RFLPs exist in those regions of the genome that hybridize to the pG3PD probe. We report here that pG3PD does detect an RFLP generated with the enzyme Taq 1.

MATERIALS AND METHODS

Chromosomal DNA was isolated from buffy coats of 10 ml blood samples obtained from the donor population within our blood service region. Nuclei were obtained from white cells during a 60 minute incubation of the buffy coat on ice in 5 mM Tris-Cl pH 7.5 containing 2.5 mM $MgCl_2$, 160 mM sucrose, and 0.5% Triton X-100. Nuclei were pelleted from the lysate by centrifugation. Chromosomal DNA was liberated from the nuclei during an overnight incubation at 37° C in 400 ul of 50 mM Tris-Cl pH 7.5 containing 24 mM EDTA, 75 mM NaCl, 1% SDS and 100 ug/ml proteinase K. Chromosomal DNA was extracted with phenol:chloroform (9:1) and then twice with chloroform:isoamyl alcohol (24:1). DNA was then dialyzed against several changes of 10 mM Tris-Cl pH 8.0 with 1 mM EDTA at room temperature to remove residual traces of organic solvents. DNA concentration was estimated on the basis of U.V. absorption at 260 nm.

Ten microgram aliquots of chromosomal DNA were digested in a 200 ul volume with 20 units of Taq 1 (Bethesda Research Labs) at 65° C for 4 hours as recommended by the supplier. DNA fragments were recovered from the digest by ethanol precipitation and centrifugation and then were electrophoresed in 0.8% agarose gels in 89 mM Tris-borate buffer pH 8.2 containing 2 mM EDTA. Following electrophoresis, gels were soaked for 10-15 minutes in 0.25 M HCl to depurinate the DNA and facilitate transfer. The gels were then soaked and blotted onto Zetabind membranes (AMF Cuno Meriden, CT) in 0.4 N NaOH. The filters were rinsed in 0.1X SSC at 65° C for 30 minutes and then air dried. Filters were pre-hybridized overnight at 42° C in 50% de-ionized formamide, 20 mM PIPES pH 6.8, 0.8 M NaCl, 2 mM EDTA, 0.1% SDS, 5X Denhardts solution, 200 ug/ml sheared, denatured salmon sperm DNA, 10 ug/ml poly A, and 10% (wt./vol.) dextran sulfate. Hybridization was performed for 20-24 hours at 42° C in the same buffer containing 5 X 10^6 cpm/ml of nick translated and heat denatured insert from the pG3PD plasmid. Blots were washed 4 times at room temperature for 10 minutes each in 2X SSC + 0.1% SDS, 2 times at 65° C for 30 minutes each in 1X SSC + 0.1% SDS, and 2 times at 65° C for 30 minutes each in 0.1X SSD + 0.1% SDS. Filters were exposed for 24-72 hours to X-ray film in cassettes containing intensifying screens.

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Restriction Site Polymorphism (RFLP) Detected with pG3PD and Taq 1.

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INTRODUCTION

Blood banks have been performing genetic testing since the discovery of the ABO blood groups by Landsteiner in 1900. Today blood banks routinely test for a number of clinically relevant blood groups and some also determine HLA phenotypes for tissue transplantation purposes. In addition, some blood banks perform genetic testing to evaluate cases of disputed parentage. Genetic markers that are analyzed in paternity cases include blood group and HLA antigens determined by standard serological methods and red cell enzymes and serum proteins analyzed by electrophoretic methods. Each of the testing methods is distinct and requires a different expertise.

RFLP (Restriction Fragment Length Polymorphism) mapping represents one of the newest methods of genetic testing. In this technique, chromosomal DNA is digested with an endonuclease specific for a particular sequence of nucleotide bases which cuts the DNA into a heterogenous family of fragments differing in size and separable by electrophoresis in agarose gels. Individual fragments within the digest can be visualized following their transfer from the agarose gel onto filter paper using Southern blotting (Southern 1975) and then hybridizing the filter to a cloned DNA probe that has been labeled in some way. The application of RFLP mapping to genetic testing relies upon the polymorphisms that exist within the population in the presence or absence of a particular restriction endonuclease recognition site in the chromsomal DNA. A single nucleotide base substitution in the chromosomal DNA can create or destroy a restriction site thus altering the size of a DNA fragment in the digest. Restriction site polymorphisms are inherited in Mendelian fashion and thus fulfill the criteria for a genetic marker. The power of RFLP mapping relates to the fact that essentially all of the chromosomal DNA can be analyzed with the technology whereas the classical methods analyze only the 1% or so of the DNA that actually encodes and expresses a gene product. Thus, a much larger pool of genetic information is available for evaluation.

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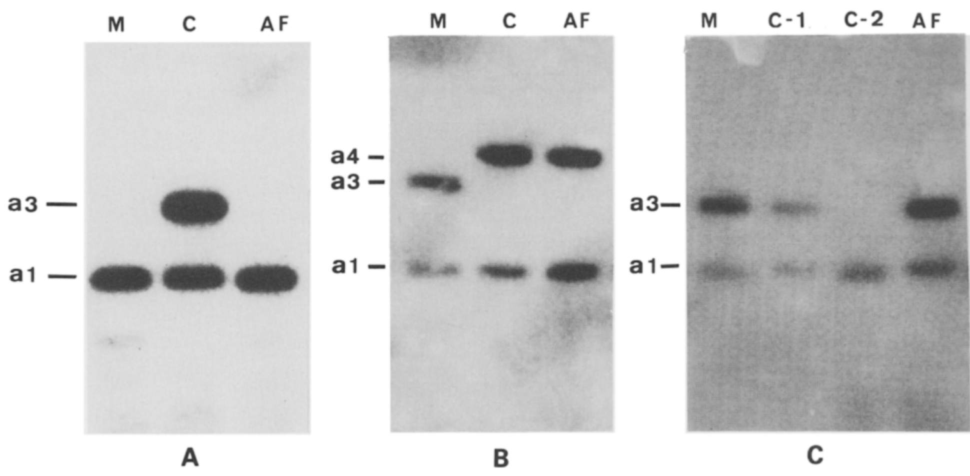

Fig. 2. Examples of paternity cases analyzed for HRAS-1 RFLP. M : mother; C : child; AF : alleged father. Panel A : a case of exclusion. Panels B and C : cases of inclusion. The probabilities of paternity were 0.9014 in Panel B, 0.5148 for child-1 and 0.3712 for child-2 in Panel C (C-1 : child-1, C-2 : child-2).

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Table 2. Parents - offsprings Combinations in HRAS-1 RFLP

parental types	No. of families	offspring types a1/a1	a1/a3	a1/a4	a3/a3	a3/a4	a4/a4	a1/a2	a2/a3	a2/a4	a2/a2
a1/a1 × a1/a1	7	16 (16)[a]	- -	- -	- -	- -	- -	- -	- -	- -	- -
a1/a1 × a1/a3	4	4 (3.5)	3 (3.5)	- -	- -	- -	- -	- -	- -	- -	- -
a1/a1 × a1/a4	3	5 (4)	- -	3 (4)	- -	- -	- -	- -	- -	- -	- -
a1/a3 × a1/a3	1	1 (0.5)	0 (1)	- -	1 (0.5)	- -	- -	- -	- -	- -	- -
a1/a4 × a1/a4	1	0 (0.75)	- -	1 (1.5)	- -	- -	2 (0.75)	- -	- -	- -	- -
a1/a3 × a1/a4	2	1 (1)	1 (1)	1 (1)	- -	1 (1)	- -	- -	- -	- -	- -
a1/a2 × a1/a3	1	0 (0.5)	0 (0.5)	- -	- -	- -	- -	1 (0.5)	1 (0.5)	- -	- -

[a] Expected numbers are given in parentheses.

Gene frequencies of these alleles were : a1 = 0.8469, a2 = 0.0016, a3 = 0.0953, a4 = 0.0546, a5 = 0.0016, $X^2 = 2.564$ $0.30 < P < 0.50$, 3 d.f. The observed phenotype distribution is in good accordance with Hardy-Weinberg equilibrium.

Pedigree Analysis of HRAS-1 Alleles

Table 2 shows Parents - offsprings phenotype combinations. The four alleles were consistent with their inheritance as Mendelian alleles.

Application of HRAS-1 Alleles to the Paternity Testing

Figure 2 shows the practical cases of paternity testing. The probability of paternity exclusion of HRAS-1 was calculated as 12.7 %, which is not very high, however, when HRAS-1 RFLP is used in addtion to the ordinary methods, such as blood types and protein polymorphisms, it would be one of useful markers. The probability of paternity and of paternity exclusion by HRAS-1 is possible to be combined with the probabilities from the ordinary methods, which will result in more accurate determination of paternity.

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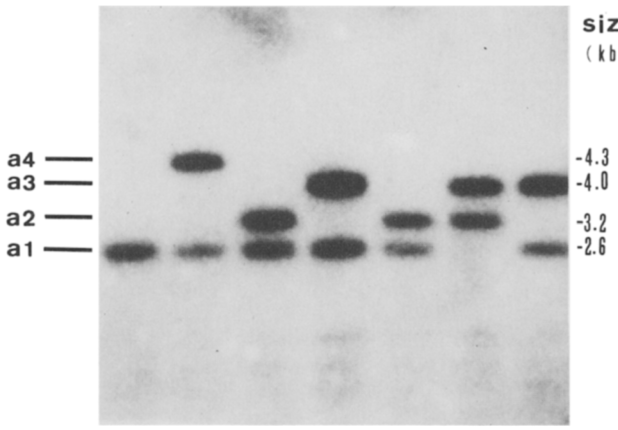

Fig. 1. Four HRAS-1 alleles (a1, a2, a3 and a4) observed in the Japanese population. Each lane shows HinfI - digested DNA from different individuals.

Table 1. Allele Frequencies of HRAS-1 in the Japanese Population and Other Ethnic Groups

Alleles	Size (kb)	Number of alleles(%)	Caucasian (Krontiris et al. 1985)	Unknown but not Japanese (Thein et al. 1986)	(Heighway et al. 1986)
a1	2.6	542 (84.6 %)	69 %	68 %	60 %
a2	3.2	1 (0.2 %)	12 %	6 %	16 %
a3	4.0	61 (9.5 %)	10 %	9 %	13 %
a4	4.3	35 (5.5 %)	6 %	13 %	7 %
other alleles		1 (0.2 %)	3 %	4 %	4 %
Total		640 (100.0 %)	100 %	100 %	100 %

The two right hand columns show frequencies reported from the United Kingdom. Baird et al. (1986) reported that the frequency of three alelles (a1, a2 and a4) was, in total, 82.4 % in Caucasians, 68.6 % in American blacks and 76.2 % in Hispanics. Previously reported allele sizes are assigned to those detected in HinfI - digested DNA.

As shown in Table 1, the frequency of a1 allele was higher in the Japanese population than in other ethnic groups, and the frequencies of a3 and a4 were similar. However, the significant difference was observed in the a2 allele, where only one allele was found among 320 Japanese (0.2 %). Besides these four alleles, rare alleles were observed which were measured as 2.5 kb and 3.8 kb in size. We include 2.5 kb allele to a1 (2.6 kb) allele because technical limitation was impossible to distinguish 2.5/2.6 from 2.6/2.6. The other allele (3.8 kb) was designated a5.

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Allelic Frequency of Polymorphic DNA Sequences in Japanese and Its Application to the Paternity Testing

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INTRODUCTION

The cellular oncogene Ha- ras-1 (HRAS-1) is localized to the short arm of chromosome 11. DNA sequence data suggest that this region consists of a variable tandem repetition (VTR) of a 28-base-pair consensus sequence at the 3' flanking region of the gene (Capon et al. 1983). Certain restriction enzymes cut sites closely outside of this tandem repetition resulting in restriction fragment polymorphisms (Goldfarb et al. 1982). The allele frequency and its application to the paternity determination, has been reported (Baird et al. 1986), and the frequencies were much different among American blacks, Caucasian and Hispanics. Here, we represent the allele frequency of HRAS-1 in the Japanese population, its pedigree analysis and application to paternity testing.

MATERIALS AND METHODS

High molecular DNA was isolated from leukocytes which had been separated by Hypaque-Ficoll method (Ferrante and Thong 1980). Approximately 10 μg of DNA was digested with HinfI and the digests were fractionated by 0.8 % Agarose gel electrophoresis. After denaturation and neutralization, DNA was transferred to a nylon membrane and hybridized with labeled DNA probe. As a probe, a 2.5 kb BamHI/SacI fragment containing VTR was nick-translated with ^{32}P-dCTP (specific activity : > 1×10^8 cpm / μg). Bands were visualized by autoradiography.

RESULTS AND DISCUSSION

Figure 1 shows the four observed alleles (a1, a2, a3 and a4) which were, when digested with other enzymes, corresponded to the common alleles previously reported (Krontiris et al. 1985; Thein et al. 1986; Heighway et al. 1986; Baird et al. 1986).

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From a total of 100 families, an exclusion rate of 79% was calculated for hMF1.

SUMMARY

The results obtained using the probe hMF1 are very largely in agreement with those obtained by conventional testing.

The cases where partial digests result in false exclusions are of obvious importance and highlight the relevance of testing in parallel with conventional systems and need for careful monitoring of digestion.

The estimated exclusion rate of 79% makes hMF1 a useful tool in parentage testing, particularly in conjunction with conventional systems.

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2. Families Showing Exclusions from Paternity on Conventional Tests

Table 3. Results of families showing exclusions on convetional Tests

	Conventional tests	hMF1 tests		
	Ex	Ex	Non-Ex	NUR
No. of families	11	6	2	3

Ex : Excluded Man
Non-Ex : Non-excluded men
NUR : No Useful Result

Table 4. Cases showing exclusion from paternity on conventional tests but no exclusion by hMF1 testing.

	Conventional systems showing exclusions	No. of paternal bands shared by man and child
Case 1	PGM (1st Order)	1
Case 2	No exclusion from 17 systems Only subsequent HLA testing showed exclusion.	6

In Case 1, where man excluded by conventional tests, only 1 band seen shared by child and PF. In Case 2, the putative father was excluded only by HLA testing and not by any of the other 17 conventional systems used. The HLA genes A2 or Ax, B27 and CW1 present in the child and of paternal origin were lacking in the putative father.

The presence of 6 bands of known paternal origin shared by the child and the putative father is surprising. The possibility of the true father being a close relative of the excluded man could explain this finding.

3. Estimation of the Power of Exclusion of hMF1

By construction of false families by using unrelated individuals on the same gel as mother and child as putative fathers, the database for exclusion statistics can be extended.

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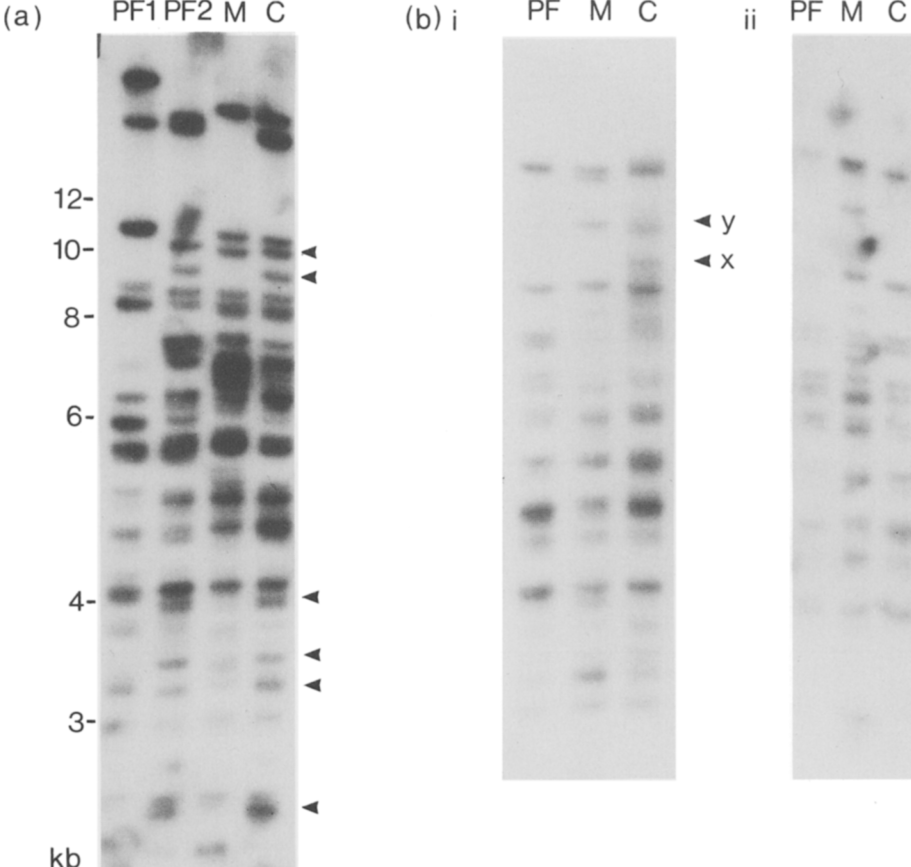

Fig. 1 (a) DNA profiles of a family in a case of disputed paternity involving 2 putative fathers (PF1, PF2), mother (M) and child (C). Paternally derived bands present in the child and PF 2 only are arrowed. These bands exclude PF1 from paternity and the large number of paternally specific bands showed by PF2 and the child strongly suggest that he is the true biological father. (b) DNA profiles of a family showing an apparent exclusion on hMF1 testing where no exclusions were found by conventional tests. In the first test (i), the child appears to show no exclusion band (x) and a maternally specific band (y), both of which are missing when the test was repeated (ii). The fuzzy background signal on the autoradiograph and the presence of DNA of higher than expected molecular weight on the gel (when visualised by ethidium bromide staining (UV transillumination) are indicative of the partial digest responsible for these artefacts.

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RESULTS

A total of 51 families were tested by both hMF1 and conventional methods. Of those, 14 gave no result by DNA testing, either as a result of insufficient DNA in one or more of the samples, or as a result of incomplete digestion of the DNA in one or more of the samples. Both of these problems have now been overcome by refinements to the method of DNA preparation.

1. Families Showing No Exclusions on Conventional Tests

Table 1. Results of families showing no exclusions on conventional tests.

	Conventional tests	hMF1 tests		
	Non-Ex	Non-Ex	A-Ex	NUR
No. of families	26	19	2[a]	5

Non-Ex : Non-excluded men
A-Ex : Apparently excluded men
NUR: No Useful Result (Mother and child showing identical band patterns)

a : see Table 2.

Table 2. Apparent exclusions from hMF1 testing

	PI from conventional tests	No. of apparent excluding bands
Case 1	155	1
Case 2	20333	1

In Case 1. the child's DNA was only partially digested and the profile showed one exclusion band and one maternally specific band, both of which dissappeared when the test was repeated. (See Fig. 1).

In Case 2, the child showed one very clear exclusion band which did not appear in either parent. The mother's DNA was only partially digested with a high background signal. When repeated the mother's DNA still did not digest to completion but the apparent exclusion band from the child was now visible in the mother.

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An Evaluation of the Use of DNA Probe hMF1 in Cases of Disputed Paternity by Comparison with a Range of Well Established Blood Group Polymorphisms

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INTRODUCTION

Recently discovered polymorphisms at the DNA level appear to offer great potential in the field of parentage testing (Jeffreys 1985a, 1985b).

DNA probe hMF1 (human Molecular Fingerprinting) was discovered in the laboratory of one of us (DIH) and its applications to parentage problems has been investigated.

In this study the results from tests using some 17 well established red cell antigen, serum protein and red cell enzyme polymorphisms are compared with results obtained using probe hMF1. Conventional systems used included ABO, MNSs, Rh, Duffy, Kell, Lutheran, Hp, EAP, Glo, PGM, Gc, ESD, ADA, AK, PLG, Tf and Pi.

Probe hMF1 is a member of the Midi-Sat family of hypervariable tandem repeats and has a repeat unit of 40bp.

METHODS

The tests for the 17 conventional blood grouping systems were carried out by standard methods.

For hMF1 testing, DNA samples for analysis were routinely prepared from 100ul of whole blood largely following the method of Gill (1985). Following digestion with a suitable restriction enzyme, in most cases _Taq_ 1, they were electrophoresed on 25cm 0.7% agarose/TAE gels at 30-50 V for 20-48 hours. The DNA was Southern blotted onto Biotrace RP nylon membrane (Gelman Sciences Ltd., Northampton, U.K.). The probe was labelled by nick translation as the isolated insert and hybridisation was carried out at 65°C overnight. Washing was generally to 0.1 x SSC at 65°C and autoradiography was for a period of 6-48 hours.

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Es zeigte sich, daß bei zwei der vier untersuchten Paternitätsfälle durch den Einsatz der vier RFLP-Systeme die gerichtlicherseits geforderte Vaterschaftswahrscheinlichkeit von W = 99,75% ohne die Bestimmung der HLA-Merkmale erreicht werden konnte.

LITERATUR

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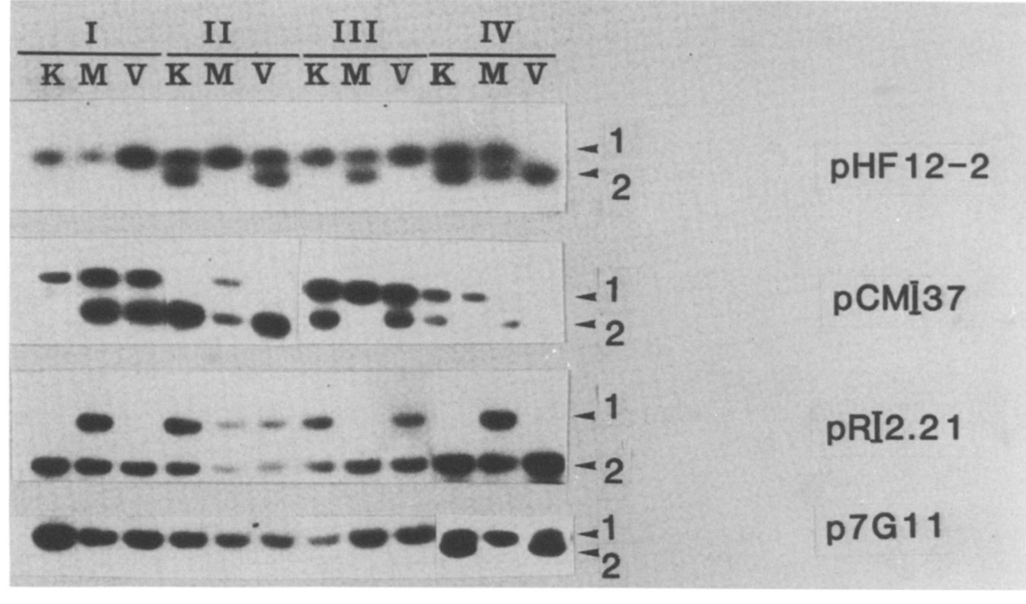

Abb.1. MspI-digested DNAs from members of four cases (I,II,III, IV) of disputed paternity hybridized with DNA probes. K = child, M = mother, V = putative father. Fragment numbers (1,2) indicated by arrows correspond to the allele numbers. The constant band present in hybridization with probe pRI2.21 is not shown.

Für jede KMV-Konstellation wurden die EM-Werte ermittelt (Essen-Möller 1938) und in die Berechnung der Vaterschaftswahrscheinlichkeit einbezogen. Die resultierenden W-Werte sind in Tabelle 3 zusammengestellt.

Tabelle 3. Plausibilities of paternity including the RFLP-systems

	W(%) - DNA-probes	W(%) + DNA-probes
Case I	85.00	94.00
II	99.66	99.93
III	99.38	99.70
IV	98.80	99.91

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Tabelle 2. DNA-probes used for paternity testing

Probe	Allele	Frequ.	Chance of non-father exclusion (%)
p7G11	1	0.88	9.44
	2	0.12	
pRI2.21	1	0.33	17.22
	2	0.67	
pHF12-2	1	0.65	17.57
	2	0.35	
pCMI37	1	0.50	18.75
	2	0.50	
		systems combined	49.80

MATERIAL UND METHODE

Aus den für die HLA-Untersuchungen gewonnenen EDTA-Bluten der Probanden wurden 1 bis 2 ml abgezweigt. Die DNA wurde aus den Lymphozyten nach Inkubation mit 0,5 % SDS und Proteinase K durch mehrmalige Behandlung mit Phenol extrahiert. Für die Spaltung mit dem Restriktionsenzym MspI wurden 5 Enzymeinheiten pro µg DNA eingesetzt. Die resultierenden Fragmente wurden elektrophoretisch im Agarose-Gel nach Länge aufgetrennt. Relevante Gel-Bereiche wurden herausgeschnitten, die Fragmente auf eine Nylon-Membran transferiert und mit den radioaktiv markierten DNA-Sonden hybridisiert. Für jede Sonde wurde ein eigener Filter verwendet. Die Filter wurden bei - 80 °C mit 2 Verstärkerfolien einem Röntgenfilm exponiert.

ERGEBNISSE

In Abb. 1 sind die Resultate der Hybridisierungen dargestellt. Auf jedem der Autoradiogramme sind zwei verschiedene Fragmentbanden zu erkennen. Sie entsprechen jeweils den zwei Allelen des polymorphen DNA-Locus, der durch die betreffende Sonde erkannt wird. Demnach tritt bei Homozygotie nur eine der zwei möglichen Banden auf. Bei Heterozygotie sind beide Banden zu erkennen. Mit keinem der vier verwendeten RFLP-Systeme wurde ein Putativvater ausgeschlossen.

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RFLP als zusätzliche Systeme in der Abstammungsbegutachtung

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SUMMARY

Four DNA-probes each detecting a two allele *MspI*-RFLP were used for paternity testing. This resulted in an increased plausibility of paternity in all the four cases tested.

In der Abstammungsbegutachtung werden am Institut für Rechtsmedizin München derzeit 28 Blutgruppensysteme routinemäßig eingesetzt. Wird die von den Unterhaltsgerichten geforderte Vaterschaftswahrscheinlichkeit W = 99,75 % nicht erreicht, werden HLA-Merkmale in die Begutachtung einbezogen.

Bei 4 Paternitätsfällen, in denen der geforderte W-Wert erst nach HLA-Typisierung erreicht wurde (Tabelle 1), setzten wir Restriktionsfragmentlängenpolymorphismen (RFLP) als zusätzliche Systeme ein.

Tabelle 1. W(%) of cases selected for RFLP-analysis

	W(%) - HLA	W(%) + HLA
Case I	85.00	99.88
II	99.66	99.95
III	99.50	99.99
IV	98.80	99.99

Dazu wurden die in Tabelle 2 aufgelisteten DNA-Sonden verwendet, die uns ausschließlich für wissenschaftliche Untersuchungen überlassen wurden. Die Sonden greifen jeweils einen *MspI*-RFLP heraus, der in zwei Allelen auftritt. Die Ausschlußchance für einen Nichtvater beträgt bei Kombination der Systeme ca. 50 %. Weitere Charakteristika der Sonden sowie ihre Hersteller sind der Literatur zu entnehmen (Human Gene Mapping Conference 8 1985).

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COMPARISON OF COST BENEFIT BETWEEN TRADITIONAL PATERNITY SYSTENS AND RFLP ANALYSIS. A pilot study.

B.Olaisen, R.Jonassen, E.S. Berg, B.Mevåg, and H.Lie. Institute of Forensic Medicine, University of Oslo, Rikshospitalet, Oslo

As the basis for a cost/benefit analysis, we have used a material including 20 paternity cases each including mother, child, putative father and two randomly chosen non-fathers.
Complete typing was performed in our basal paternity dianostics set-up including ABO,Rh,MN,K,Gc(subt.),C3,Hp(subt.),GLO,ESD, GPT,ACP and PGM (subt.) systems.
The material was also typed with restriction fragment length polymorphisms (RFLP) analysis with combined XBaI/BglII restriction enzyme digestion and hybridization with C4 and HLA-DR probes.
Estimation of costs included technician's wages and cost of necessary reagents etc. By comparison, the protein typing set-up is two to three times more cost-consuming than the RFLP analysis. The efficiency of the two procedures were about equal. The main conclusion is that RFLP analysis using a proper choice of probes, is extremely promising for use in future paternity diagnostics.
Preliminary results of DNA fingerprinting with M13 probe (Hinf1 digestion) in the same material are included.

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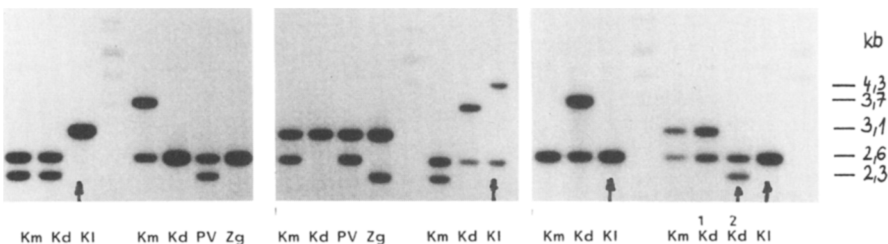

Abb. 6 HRAS, Vaterschaftsausschlüsse (Pfeil) in 6 Gutachten

Bei nur geringfügig verbesserter Trenntechnik kann man mit weit höherer Aufklärungsrate rechnen.

Für die praktische Anwendung hat man die Wahl zwischen folgenden Systemen: 1. mehrere Zweiallelen-Loci (Smouse and Chakraborty 1986), 2. hochpolymorphe Marker mit 10-20 Allelen und 3. Marker, die dem 1985 von Jeffreys beschriebenen Minisatellitensystem entsprechen.

Abgesehen davon, daß der Identitätsbestimmung eine theoretisch unbegrenzte Zahl von Markern zur Verfügung steht, hat die DNA-Analyse gegenüber der bisherigen Verfahrensweise folgende Vorteile: 1. das zu untersuchende Probenmaterial (DNA) ist wesentlich stabiler als das für alle bisher verfügbaren Analysen angewandte, 2. das Problem der "stummen" Gene existiert nicht und 3. selbst bei Anwendung einer großen Zahl von verschiedenen Markern ist die im Labor verwendete Technik immer die gleiche, mit der Folge einer methodisch erhöhten Sicherheit und Vergleichbarkeit.

Wir leiten aus unseren Ergebnissen den Schluß ab: Für Marker des hier beschriebenen Typs kann man mit vertretbarem Aufwand die formalgenetischen Grundlagen für eine Anwendung in der Abstammungsbegutachtung schaffen; sie können eine wirksame Ergänzung des zur Zeit verwendeten Instrumentariums insbesondere bei der Klärung von Problemfällen sein.

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Als wesentlich problematischer erwies sich der D14S1-Locus. Durch Mischen von DNA-Proben verscheidener Personen und durch jeweils mehrfach wiederholte Messungen konnten wir rd. 20 Allele unterscheiden (in der vorgenannten Arbeit sind es noch weit mehr). Der Meßfehler bei der Fragmentgrößenbestimmung war größer bzw. die Auflösung des Trennsystems war kleiner als es für eine exakte Frequenzbestimmung der einzelnen Allele nötig gewesen wäre. Die Form der sich ergebenden Verteilung läßt jedoch deutlich erkennen, daß ihr distinkte Allele zugrunde liegen, denn sie entspricht ziemlich exakt den von Baird et al. ermittelten Werten. Diese Autoren liefern noch einen Hinweis darauf, daß es sich tatsächlich um eine große Anzahl unterschiedlicher Allele handelt; es ist nämlich gezeigt worden, daß sich die entsprechende Verteilung bei anderen ethnischen Gruppen signifikant von der auch in diesem Beitrag behandelten kaukasischen Bevölkerung unterscheidet.

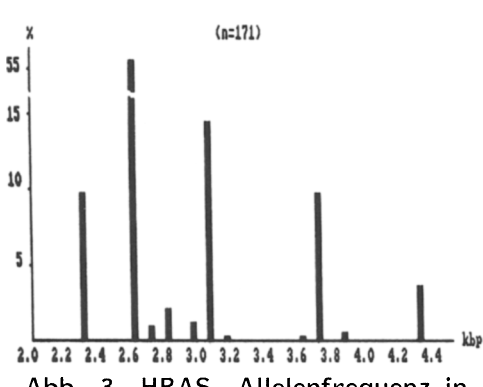

Abb. 3 HRAS, Allelenfrequenz in der deutschen Bevölkerung

Die Frage der Vaterschaft in 86 gerichtlichen Gutachtenfällen (Ein- und Mehrmann-Sachen) wurde durch Verwendung von 25 verschiedenen Blutgruppensystemen und teilweise zusätzlich durch HLA- A,B,C eindeutig geklärt. Insgesamt konnten serologisch 41 Männer von der Vaterschaft ausgeschlossen werden. Bei 22 dieser Nichtväter erbrachte die DNA-Analyse ebenfalls einen Ausschluß. In 12 weiteren Fällen waren die Ausschlüsse wegen der nicht eindeutigen Trennbarkeit der jeweiligen Allele zweifelhaft (11mal im D14S1-System, einmal im HRAS-System). Auf der anderen Seite konnte bei den nicht ausgeschlossenen Männern in keinem einzigen Fall ein widersprüchliches Ergebnis zwischen konventioneller Begutachtung und DNA-Analyse beobachtet werden.
Die Abb. 5 und 6 zeigen die genetische Situation in 11 Gutachten mit 7 Vaterschaftsausschließungen.

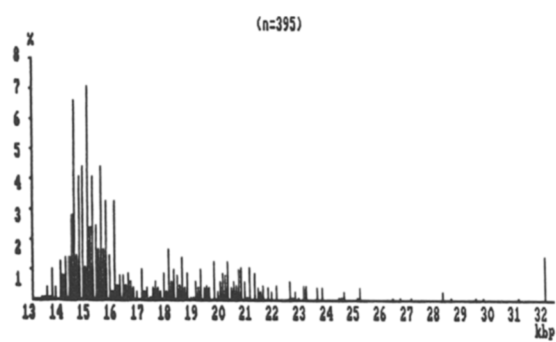

Abb. 4 D14S1, Allelenfrequenz in der deutschen Bevölkerung

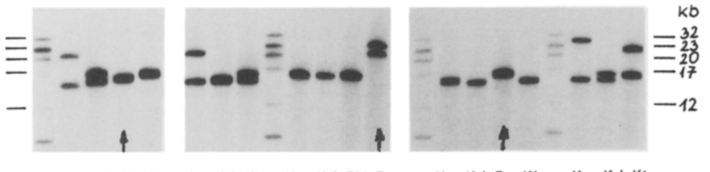

Abb. 5 D14S1, Vaterschaftsausschlüsse (Pfeil) in 5 Gutachten

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Die Auswertung erfolgte von Hand. Zur Ermittlung der Meßgenauigkeit fertigten wir aus den unterschiedlichen Größenbereichen der Allele mehrere Gele an; die Standardabweichung bei 20kbp war 1,1%, bei einem 14,5 kbp Allel 1,3%. Das optische Auflösungsvermögen in unserem Trennsystem war im Bereich der kleineren Allele (14-15 kbp) ca. 150 bp und bei den größeren (>23 kbp) ca. 1 kbp.

ERGEBNISSE UND DISKUSSION

Im Fall des D14S1-Locus haben wir in 39 Familien mit insgesamt 205 Personen und bei HRAS in 24 Familien mit 129 Personen in keinem Fall eine Ausnahme von Mendelscher Vererbung gefunden. Daraus leiten wir die Annahme ab, daß es sich um genetisch stabile Systeme handelt (Abb. 1 und 2).

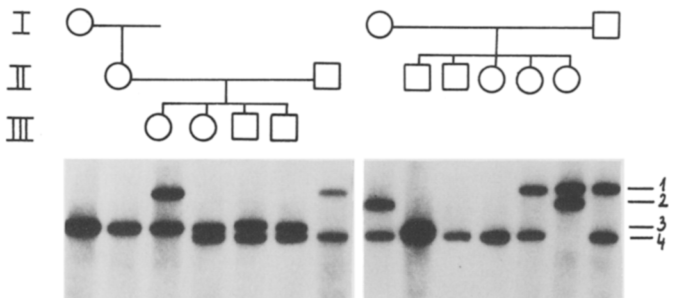

Abb. 1 D14S1, Eco RI-Polymorphismus in 2 Familien

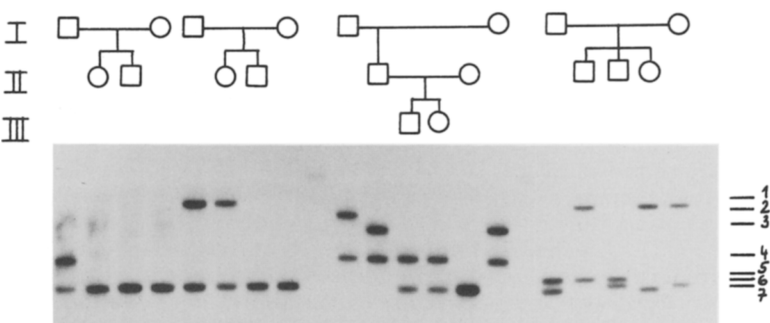

Abb. 2 HRAS, Taq I-Polymorphismus in 4 Familien

Die Untersuchung der Allelenfrequenzen bei HRAS ergab die in der Abb. 3 dargestellte Verteilung. Es sind 171 nicht verwandte Personen untersucht worden. Wir fanden 11 problemlos zu differenzierende Allele, 7 weniger als in der 1986 von Baird et al. publizierten Studie. Aufgrund der Genfrequenzen errechnete sich eine AVACH von 27,88 %.

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EIN VERGLEICH VON 25 BLUTGRUPPENSYSTEMEN MIT POLYMORPHEN DNA-MARKERN IN DER VATERSCHAFTSBEGUTACHTUNG

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Seit etwa 1975 erfolgt in der Humangenetik eine geradezu revolutionäre Entwicklung, indem sie sich die Erkenntnisse und Methoden der Molekularbiologie zueigen macht. Anfang der 80er Jahre wurde bei der Erforschung der Struktur des menschlichen Genoms eine für die Identitätsbegutachtung wesentliche Erkenntnis gewonnen; auf DNA-Ebene ist eine fast unbegrenzte Vielfalt von Polymorphismen zu erwarten. Nachdem etwa seit 1980 das Arbeiten mit der rekombinanten DNA-Technologie große Verbreitung in humanbiologisch orientierten Laboratorien gefunden hatte, sind tatsächlich bis zur sogenannten Human Gene Mapping Conference in Helsinki 1985 rd. 500 dieser DNA-Polymorphismen beschrieben worden. Ziel dieser weltweiten Bemühungen war es, das menschliche Genom möglichst dicht und möglichst gleichmäßig mit einem Netz solcher Restriktionsfragment-Längenpolymorphismen (RFLP) zu überziehen. Man wollte sich damit in die Lage versetzen, auch genetische Erkrankungen, deren eigentlicher Gendefekt nicht bekannt ist, im Genom zu lokalisieren und zu diagnostizieren. Das Konzept hat sich hervorragend bewährt; zahlreiche bedeutende Probleme der humangenetischen Diagnostik (vor- und nachgeburtliche Diagnostik) sind in den vergangenen drei Jahren gelöst worden.
Die Anwendung von DNA-Polymorphismen in der Identitätsbegutachtung, der Spurenkunde und der Abstammungsbegutachtung ist naheliegend. Im Rahmen unserer Studie haben wir 724 DNA-Isolierungen aus EDTA-Bluten und 1435 DNA-Analysen durchgeführt. Die Untersuchungen an 195 Blutspendern, 39 deutschen Familien (Raum Köln/Düsseldorf) und 85 Gutachtenfällen hatten das Ziel, die Brauchbarkeit von DNA-Sonden zu prüfen und Allel-Frequenzen für die formale Genetik zu ermitteln.

METHODEN

Der Eco RI-Polymorphismus an D14S1 läßt sich durch die von Wyman et al. 1980 beschriebene DNA-Sonde pAW101 nachweisen. Der Taq I-Polymorphismus des HRAS-Onkogens wird durch ein 879 bp langes SphI-Cl aI-Fragment aufgedeckt (Capon et al. 1983). Die Sonden wurden uns freundlicherweise von den Drs. White und Capon zur Verfügung gestellt. Die Darstellung der Allele erfolgte mit der üblichen Southern-Blot-Prozedur. Wir verwendeten extrem langlaufende 0,6%ige Agarosegele für den D14S1-Polymorphismus und 1,2%ige Gele für das HRAS-Onkogen. Die aus dem Plasmid herausgeschnittenen Sonden wurden per Nick-Translation bis auf rd. $2x10^8$ cpm/μg radioaktiv markiert. Die Expositionsdauer betrug zwischen 10 und 16 Stunden. Zur Messung der Allelengrößen wurde jeweils neben der DNA der Probanden käufliche und/oder zusätzlich restriktionsverdaute Längenstandard-DNA auf das Agarosegel gebracht. Zusammen mit der radioaktiv markierten Sonde wurde jeweils eine kleine Menge ebenfalls markierte λ-DNA für die Hybridisierungslösung verwendet.

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Figure 1.

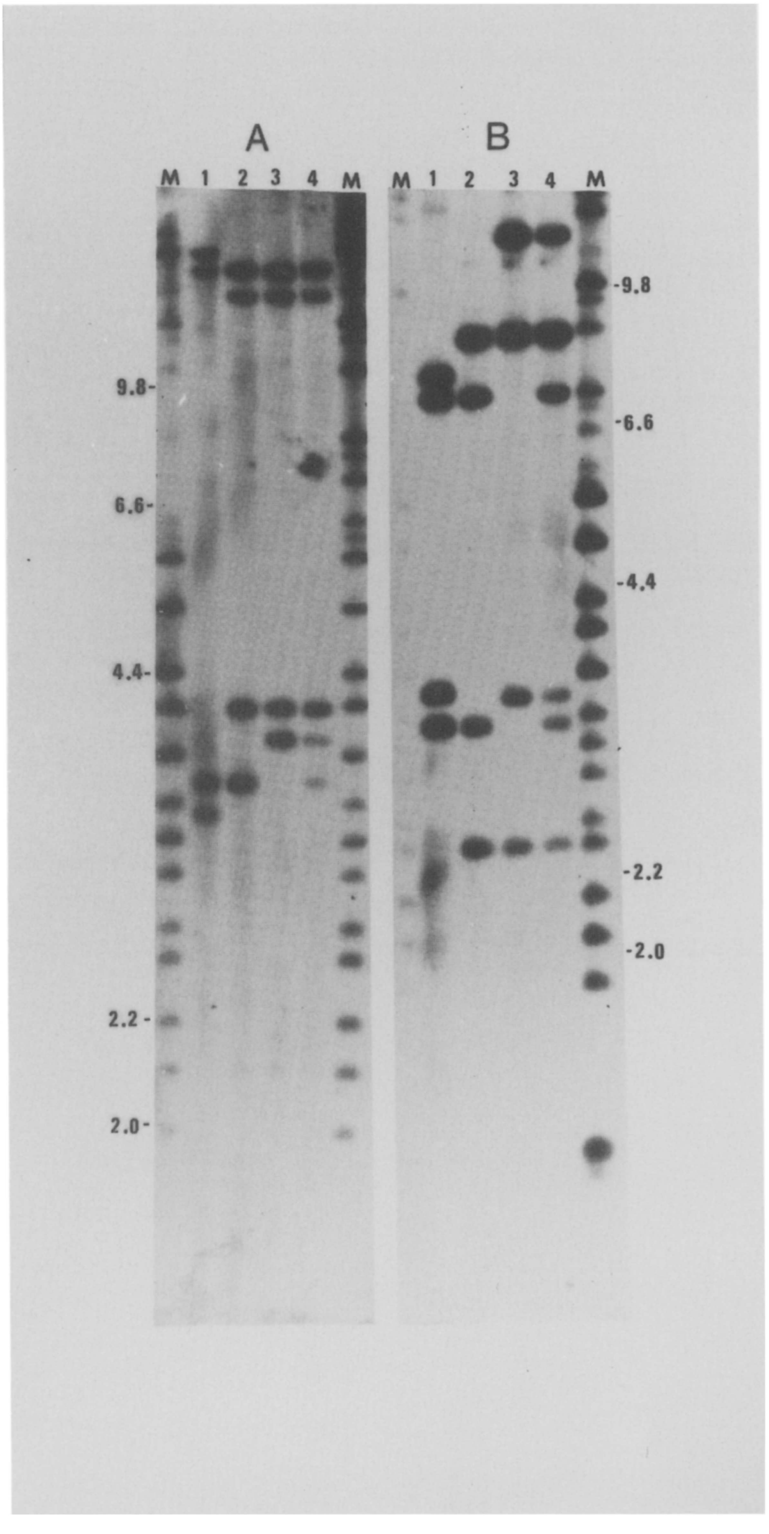

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Figure Legends

Figure 1. Example of DNA-Print for a paternity inclusion.

DNA digested with Pst 1 was hybridized with 4 DNA probes recognizing hypervariable loci. Lane 1, DNA from the mother. Lane 2, DNA from the child. Lane 3, DNA from the alleged father. Lane 4, Mixture of DNA from the child and alleged father. (A). Pattern with probes pAC255 and pAC256. (B). Pattern with probes pAC061 and pAC222.

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Table 1. Probes used for the analysis of Pst 1 digested DNA: number of alleles and median P_I.

Probe	Number of Alleles	Median P_I
pAC061	>80	13.14
pAC222	15	2.24
pAC255	>80	13.39
pAC256	>30	9.23
	Combined P_I: 3638	

With a combination of probes such as the one listed above the median P_I was in 50% of cases, greater than 3600. This corresponds to a probability of paternity (W) greater than 99.97%, using a 0.5 prior probability. In all paternity cases where the 4 DNA probes included the alleged father, the P_I was greater than 100.

An exclusion was indicated when the child contained DNA bands not present in the alleged father. This was confirmed by fractionating in a single lane an equal mixture of DNA from the child and the alleged father. Two bands were considered of different size if they did not co-migrate in this mixture lane. All cases of exclusion, of an alleged father, were observed with at least 2 of the 4 probes used for testing. The highest P_I obtained in cases of exclusion, with the probe(s) that did not exclude was 49.3. The exclusion probability, or the efficiency of the system in eliminating falsely accused fathers was calculated for each probe using the allele frequency database and the formula described for RFLPs by Ito et al. (6)(Table 2). Therefore the analysis of paternity cases with these 4 probes is expected to exclude approximately 99.5% of falsely accused males.

Table 2. Average power of exclusion with four hypervariable loci.

	DNA probes				
	pAC061	pAC222	pAC255	pAC256	Total
Ethnicity					
Blacks	0.77	0.56	0.83	0.81	0.9967
Caucasians	0.58	0.44	0.88	0.79	0.9940

In conclusion, the use of DNA probes that detect highly polymorphic loci is an extremely powerful system for the analysis of cases of disputed paternity.

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Pst 1 digested DNA samples from paternity cases were loaded on agarose gels with mother, child, alleged father, and a mixture of child and alleged father flanked by size standards composed of bacteriophage lambda and phi X174 DNAs cut with various endonucleases. A total of three gels were generated for each case by two individuals. The gels were hybridized with different combinations of probes to provide duplication and allele assignment in cases where DNA probe alleles might overlap.

Paternity Determination and Calculation of the P_I.

The size of the bands detected with each DNA probe was determined by comparing the relative mobility of the restriction fragments to that of DNA size standards. To ensure accuracy in size determination a fitting procedure described by Elder and Southern (5) was incorporated into a computer program that determines the best fit for the standards.

A database of allele frequencies, for American Blacks and Caucasians, has been established with each of the four DNA probes and used in the P_I calculations. The frequency of a particular DNA band size, co-migrating between father and child, was derived from these databases, taking into account the standard deviation in band size determination and resolution of the gel (manuscript in preparation).

Results and Discussion

The average amount of high molecular weight DNA isolated from blood samples was about 50 micrograms. Each sample was processed by two individuals independently. After digestion, each sample was ethanol precipitated to concentrate the sample and redigested. Approximately 0.5 ug of each digestion was evaluated for completeness of digestion by electrophoresis. The diagnostic gels were prepared for maximal resolution in the size range from 2.0 kb to 20.0 kb. After transfer to a nylon membrane, the filters were hybridized with combinations of two probes which recognize highly polymorphic loci (Table 1). The size range of the bands generated with pAC255 and pAC256 did not overlap and they could be unambiguously assigned to their respective locus (Fig 1A). Probe pAC255 hybridized to DNA fragments within the size range of 7.0 kb to 25.0 kb while the probe pAC256 annealed to fragments within the range of 2.0 to 5.0 kb. The alleles recognized with pAC061 and pAC222 ranged in size from 3.0 kb to 25.0 kb and 1.6 to 5.0 kb respectively (Fig 1B). In the example presented in this figure the P_I was 4376 and the probability of paternity (W) 99.98%. To resolve potential overlap for this last combination of probes and as a duplication of sample processing, a third blot was hybridized to pAC061 and pAC255 (results not shown). The analysis of the results of these three blots allows the unambiguous assignment of the alleles corresponding to each polymorphic locus. This system also allows the duplication of testing by having duplicate results on probes pAC061 and pAC255.

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THE APPLICATION OF DNA-PRINT FOR THE ESTIMATION OF PATERNITY.

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Abstract

We have applied the method of DNA polymorphism analysis to resolve paternity cases. DNA samples were isolated from the blood of mother, child, and alleged father, digested with the restriction endonuclease Pst 1, size separated by agarose gel electrophoresis, and hybridized with four recombinant DNA probes which recognize hypervariable regions in the human genome. Determination of DNA fragment sizes was accomplished with a computer assisted digitizing system. Co-migration of paternally derived DNA fragments in the child and alleged father indicated possible paternity, and a paternity index (P_I) was calculated from allele frequency tables accumulated for each polymorphic locus. The cumulative power of exclusion with these four probes, for American Blacks and Caucasians, was on average 99.5% and the mean paternity index more than 3600.

Introduction

Genetically inherited differences among individuals can be visualized at the DNA level as restriction fragment length polymorphisms (RFLPs)(1,2). The most polymorphic DNA regions are those containing short tandem repeats of a short DNA sequence (3). The present report discusses the use of a combination of four such DNA probes to determine biological paternity in disputed parentage cases.

Materials and Methods

DNA Purification and Probes

High molecular weight DNA was isolated from 1 ml of peripheral blood by standard procedures (2) from samples sent to Lifecodes Corp. for paternity determinations. Five micrograms of each DNA sample was digested twice with a five-fold excess of Pst 1 (Bethesda Research Laboratories) restriction endonuclease and size separated by electrophoresis in an 0.9% agarose gel. After transfer to a nylon membrane and hybridization with ^{32}P-labelled recombinant DNA probes, the filters were washed and exposed to X-ray film (2).

Four DNA probes were used for this study: pAC061 (this probe detects the major hypervariable region of the D14S1 locus), pAC222 (clone derived from pLM0.8)(2), pAC255, and pAC256. Each probe recognizes an independent single locus. Each DNA probe was isolated as a lambda clone from a total human genomic library and following subcloning, propagated as an insert in the cloning vector Bluescribe (Promega). All probes were used as purified inserts. DNA probes were labelled using a random oligo priming reaction (4).

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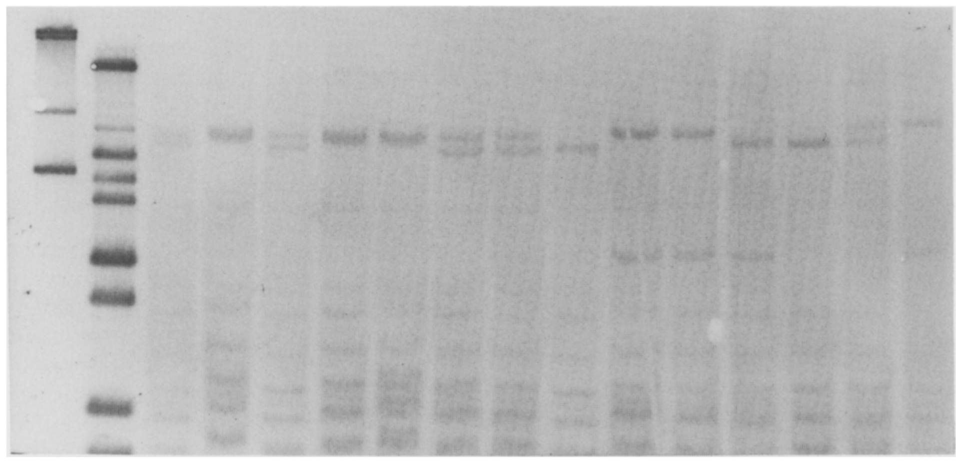

Fig. 3a Probing with L-892. Size marker controls on far left and right.

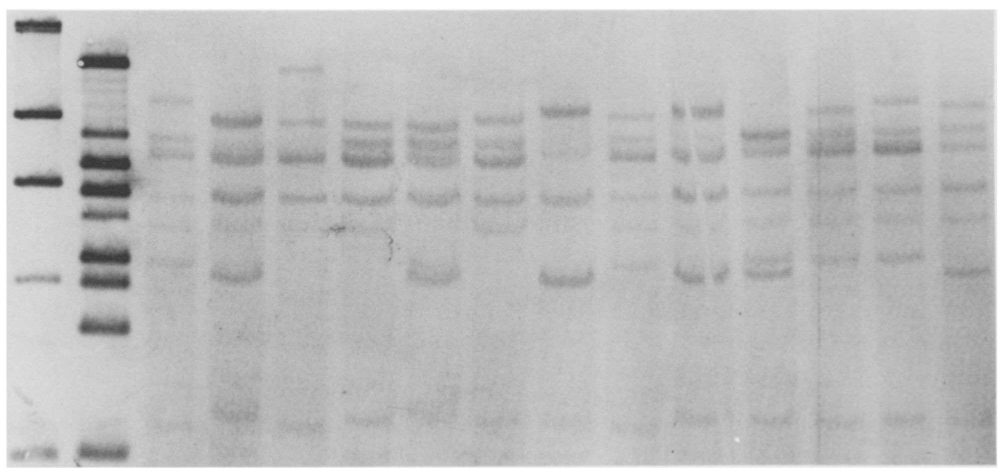

Fig. 3b Reprobing of membrane from Fig. 3b using probes cMetH, cMetD, 523-10B and pS194. Reason for darker size markers is a different source of biotinylated - λ .

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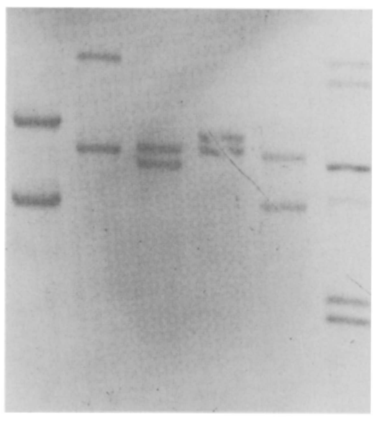

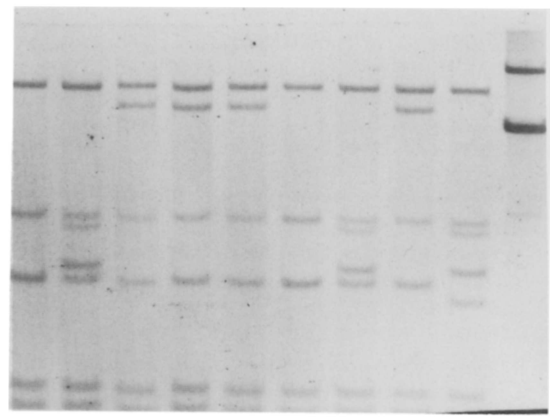

Fig. 1a pS 194 probing with size markers on far right.

Fig. 1b L-45 probing with size markers on far right.

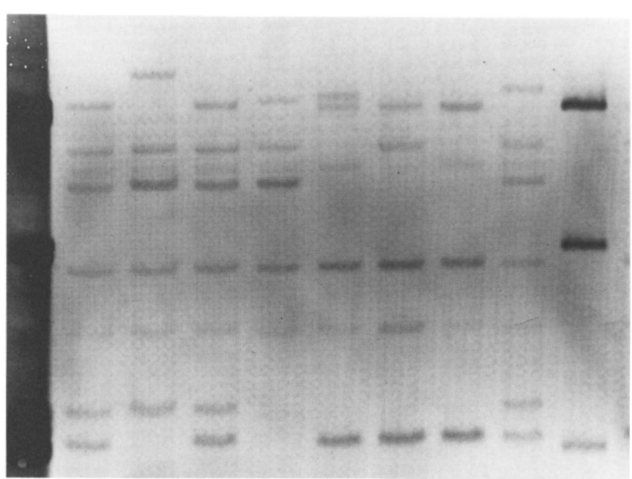

Fig. 2 Multiple probing with cMetH, cMetD, 523-10B and pS194. Size markers on far right and left.

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Membranes were also rehybridized up to 4-5 times without a noticeable loss of signal. This permitted us to return to earlier hybridized membranes and study older paternity cases and population studies, figure 3.

As expected the observed rate of exclusion varied with the "P" value of the individual probes. Multiple simultaneous probings, such as 228C and 513-5H after MspI restriction or cMetH and 523-10B, sometimes included with pS-194 after TaqI restriction provided the anticipated number of combined exclusions, table 3. Some of the variation from expected values was due to the small sample size. No additional exclusions were observed in cases with PI ≥ 100. In cases with multiple exclusions by our normal test battery we observed that the putitive father was excluded 7/8 times when using L-45 and L-892 and pS 194.

In conclusion the use of biotinylated probes permits easy and inexpensive analysis of RFLP's in parentage testing. The substrates are not a biohazard, are stable upon storage, give clearly resolved bands and are easily adapted to the normal laboratory setting.

TABLE 3
RESULTS OF BIOTINYLATED PROBES ON PATERNITY CASES

Probe	# cases Tested	# Exclusion Other Systems	# Exclusion with Probe	Observed Excl. Rate	Expected* Excl. Rate(P)
L-45	43	8	3	0.43	0.38
L-892	42	13	8	0.62	0.48
PS-194	46	16	12	0.75	0.67
pPW228C	108	29	6	0.20	0.18
513-5H	89	25	8	0.32	0.17
523-10B	47	11	2	0.18	0.19
518-1R	23	7	2	0.29	0.43
cMetH	71	19	5	0.26	0.18
cMetD	15	6	0	-	0.12

* Initial battery of tests included 14-16 markers with P ≅ 0.98. This value is close enough to 1.00 that "P" for each marker system should approximate observed rate of exclusion if sampe size is large enough.

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TABLE 2

GENE FREQUENCIES AND EXCLUSION PROBABILITIES DETECTED WITH PROBES

Probe	No. Tested	Alleles	Size	Gene Freq.	Exclusion Prob (P)
228C	474	A1	4.6	0.62	0.18
		A2	7.8	0.38	
513-5H	407	A1	3.4	0.67	0.17
		A2	2.1	0.32	
523-10B	277	A1	7.9	0.51	0.19
		A2	5.5	0.49	
518-1R	208	A1	2.45	0.224	0.43
		A2	2.41	0.293	
		A3	2.33	0.415	
		A4	2.25	0.050	
		A5	2.33	0.012	
		A6	2.35	0.005	
		A7	2.60	0.001	
cMetH	323	1	7.7	0.57	0.18
		2	4.0	0.43	
cMetD	135	1	6.3	0.83	0.12
		2	4.7	0.17	
L-45	171	1	7.4	0.336	
		2	4.0+3.0	0.342	
		3	3.8+3.2	0.281	0.41
		4	3.95+2.8	0.041	
L-892	154	1	9.7	0.045	
		2	9.2	0.341	
		3	8.8	0.247	0.48
		4	8.2	0.308	
		5	9.2+4.5	0.042	
		6	8.6	0.017	
PS 194	154	1	14	0.023	0.67
		2	12.3	0.003	
		3	11.1	0.006	
		4	10.7	0.039	
		5	10.5	0.042	
		6	10.3	0.049	
		7	10.2	0.032	
		8	10.1	0.078	
		9	10.0	0.019	
		10	9.9	0.206	
		11	9.6	0.081	
		12	9.4	0.062	
		13	9.2	0.019	
		14	9.1	0.006	
		15	8.9	0.003	
		16	8.6	0.332	

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TABLE 1 (CONTINUED)

Probe	Size	Vector	Enzyme	Source*
L-45	3.2	Lambda	MspI	CR
L-892	12.6	Lambda	TaqI	CR
pS-194	1.4	pUC-8	TaqI	CR
cMetH	1.6	insert	TaqI	OI
cMetD	1.1	insert	TaqI	OI

*IG = Integrated Genetics
CR = Collaborative Research
OI = Oncor Inc.

The type of membrane is vital to the use of non-isotopic probes. Nylon worked best, with Genetran-45® and Sure blot® showing the most reproducable results. Sure Blot, however, retained the most DNA after transfer and demonstrated the clearest background. Excessive blocking and the use of carrier DNA was found to be unnecessary. Carrier DNA non-specifically reacted with some enzyme conjugates and caused unwanted back-ground.

Alkaline phosphatase conjugates provided greater sensitivity when compared to streptavidin-horseradish peroxidase conjugates. Although the latter is faster it often produced excessive background if not carefully watched. It was very light sensitive and losses approximately 20-25% of band intensity when dried.

When such parameters as membrane type and source, form of enzyme conjugate and probe size are optimized the biotinylated probes easily detect single copy RFLP's when 3-5 ug of total human genomic DNA are applied to the gel. Dot blots are capable of detecting 0.1-0.5 pg of target DNA. The stability of these non-isotopically labeled probes was at least 18 months. This enabled us to use the same quality controlled probe over extended periods of time. Isotopic probes using p32 have a short half-life and require labeling probes every 10-14 days.

Table 2 lists the observed gene frequencies of the RFLP's observed in this study using the various single copy human DNA probes. Examples of single probing are shown in figures 1a and b. For two allele probes with exclusion probabilities of 0.15 - 0.19 we found it more practical to do multiple probings if overlapping of bands was not a problem, figure 2. For more polymorphic probes such as 518-1R and PS-194 we found that the non-isotopic approach demonstrated additional alleles. The better resolution obtained with this technique and hence the detection of more variants is a result of confining the signal in a narrow region versus the more diffuse signal generated by the isotopic approaches.

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both an alkaline and high salt transfer buffer. Membranes were baked at 80°C for 20-30 minutes after transfer. Listed below, with minor modifications, is the hybridization and staining procedure we described in 1986.

1. Block membrane 30-45 minutes at RT using pH 7.5 TBS (0.1M Tris-HCl, 0.5M NaCl), 1% Hammarsten casein, 3% Liquid Hipure Geletin and 0.05% Tween-20.
2. Hybridize at 42°C overnight using 20-40 ng probe/ml hybridization buffer.
3. Wash 2-3 minutes at RT in 0.16 X SSC, 0.1% SDS.
4. Stringency wash at 50-60°C in 0.16 X SSC, 0.1% SDS.
5. Wash 2 X 3 minutes in 2 X SSC.
6. Block membrane for 15 minutes (#1)
7. Prewash in complex dilution buffer of pH 7.5 TBS, 5% Tween-20.
8. Treat with streptavidin-biotin-alkaline phosphatase (Enzo Biochem or Oncor, Inc.) in complex dilution buffer for 20 minutes at RT.
9. Blot membrane and wash 3 X 5 minutes in pH 7.5 TBS buffer.
10. Wash 5 minutes in 0.1 M TBS pH 9.1 stain buffer.
11. Stain membrane on agarose stain plates.
12. Wash briefly in tap water and thoroughly dry in dark.

Membranes were rehybridized by first removing the labeled probe followed by two brief washes in dimethlyformamide to remove the bands and two washes of 2 X SSC for 4-5 minutes. Membranes were then reblocked as above and rehybridized.

RESULTS AND DISCUSSION

Purified probe inserts and probes in plasmid or lambda bacteriophage were all capable of providing good signal after nick translation with biotin. Although a large number of probes were analyzed we concentrated on the probes shown in Table 1. There appeared to be a consistent difference in signal obtained from some of the probes which did not seem to be related to the size of the probe insert. This phenomenon also holds true for probes labeled with p32 (personnal communication with labs providing probes). For example probe pPW228C at 1500 bp provided a better signal than pPW513 at 3200 bp when applied at equal concentrations to the membrane.

TABLE 1 PROBES USED IN STUDY

Probe	Size	Vector	Enzyme	Source*
pPW228C	1.5	pBR328	MspI	IG
pPW513-5H	3.2	pBR322	MspI	IG
pPW523-10B	6.5	pBR322	TaqI	IG
pPW518-1R	3.2	pBR322	XbaI	IG
cMetH	1.6	pBR322	TaqI	IG
cMetD	1.1	pBR322	TaqI	IG

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Identifying DNA RFLP'S in Routine Paternity Cases: Non-Isotopic Methods of Detection.

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An ever increasing number of polymorphic DNA probes have been reported in the literature. Thus it should be theoretically possible to select from a broad range of probes to be used in cases of disputed parentage. However, the lack of availability of many probes from the commercial sector, the general view that probes must be labeled with isotopes and various technical problems inherent with the techniques have hindered the widespread use of RFLP's for this application.

For this study we will describe data on the use of non-isotopically labeled probes for detecting RFLP's in paternity cases. Probes were labeled with biotin and subsequently detected with alkaline phosphatase conjugates using modifications of the techniques of Leary et al. (1983) and Dykes et al (1986). The probes were obtained either in plasmid vectors, bacteriophage lambda or as inserts from three commercial companies and labeled in our laboratory. The technique permitted the use of probes labeled up to 18 months prior to hybridization. Rehybridization of membranes a minimum of 4-5 times with non-isotopically labeled probes was possible. Speed, cost effectiveness and reproduceability are all important factors which make this technique well suited for general use in the area of parentage testing.

MATERIALS AND METHODS

DNA extracts used in this study were obtained from paternity cases routinely tested in our laboratory for 14-16 genetic marker systems (RBC antigens, enzymes and serum proteins). Single copy human probes used in this study were obtained from Integrated Genetics, Framingham, MA. Collaborative Research Inc, Bedford, MA. and Oncor Inc. Gathersburg, MD. Probes were used only if they had extensive family data to document inheritance, were characterized for chromosomal location and in most cases documented in the literature.

Probes were labeled with a biotin-11-dUTP nucleotide by nick translation as described by Dykes et al. (1986). Labeled probes were stored at 4°C for up to 18 months prior to use. Length of electrophoresis depended upon the probe being used. Southern blotting generally was accomplished in 2-3.5 hours for gels 6-8 mm thick. For this study we tried many membranes and finally settled on a nylon membrane from Oncor, Inc. called Sure Blot® which provided the best signal to noise ratio of any membranes tested. The membrane worked well in

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selected DNA (approximately 500 bp). Inclusion of proteinase K in the wash procedure (Wash B) was found empirically to reduce background autoradiagraphic signals.

The probe 228S has been sequenced and classified by major sequence data banks as closely related to human Satellite III DNA. It is characterised by its high proportion of the tandem repeat TTCCA. The sequence 142F has been similarly examined and classified as homologous to human alphoid (centromeric) DNA.

The 3.4 kb Y specific fragment is believed to be composed of tandem repeats, with common and clustered EcoRl and HaeIII restriction sites, but where the intervening sequences are relatively diverged (Cooke et al, 1983). One of the sequences common to these repeats is Satellite III related (Beauchamp et al, 1979). It should be noted that it is the size of the repeat fragments which is considered to be the Y specific feature, though some short base sequences within any repeat may be male specific. The presence of the other Satellite III sequence related fragments detected in both male and female DNA provides an internal reference as to the quantity and quality of DNA under examination. This is of particular advantage in the analysis of forensic specimens. In such samples, the absence of a male specific response using a "sequence specific" probe (Tyler et al, 1986) could be explained by there either being insufficient DNA recovered or the sample being female DNA. The type of patterns produced here provide a means of resolving this ambiguity, paticularly if this evidence is supported by reprobing with other repetitive sequence probes. This advantage is especially important given that although the 3.4 kb fragment occurs in approximately 5000 copies on the Y chromosome of most males, the quantity of heterochromatin from which it is derived in the DYZl region is variable, and may even be absent in some "normal" males (Goodfellow et al, 1985). Other Y chromosome fragments have therefore been argued as more reliable indicators of sex (Goodfellow et al, 1983). However these are generally of far lower copy number, reducing their forensic usefulness. Provided results are presented with the qualifications outlined, the method here provides a very sensitive means of sexing human tissue in forensic samples.

The Taq l fragment patterns have the potential for forensic use in the discrimination of individuals, either from somatic or germ tissue. Again, the ability to reprobe the same samples with the centromeric probe 142F to establish the equality of the restriction is a particular advantage on forensic samples.

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recovery of DNA agarose gels could not be reproduced by us, the best efficiency being of the order of 70% for material in 20 kb to 0.5 kb range. It is possible that a more prolonged acid treatment of the gel would provide greater depurination of double stranded DNA, hence greater fragmentation and better transfer. However an anticipated limitation might be lower hybridisation efficiencies. This may explain the improved results obtained by our method of brief acid treatment, denaturation in situ (0.4 M NaOH) and Southern blotting in dilute alkali (0.02M NaOH or less). Too dilute alkali transfer might decrease the (covalent) binding of single stranded DNA to the membrane, although transfer solutions as dilute as 4 mM NaOH still provided very satisfactory binding.

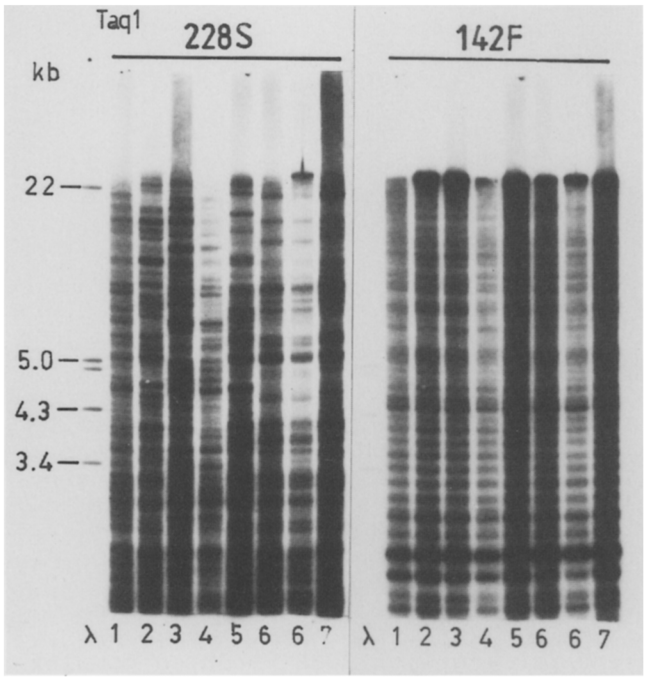

Fig. 2 Taq 1 analysis with probes 228S and 142F of individuals 1 - 7. Persons 1 - 4 are related, 5 - 7 are unrelated and duplicate 6 is sperm/hair tissue compared (3 ug DNA per sample).

The preparation of the hybridisation probe is particularly simple. The large size of the M-13/insert probe (approximately 7.5 kb including the insert 228S of 332 bp) does not seem to be inhibitory to hybridisation with either large or small DNA fragments attached to the membrane. Preliminary experimentation has shown that this probe design gave a ten-fold better autoradiographic signal when compared with a probe made by replication of the insert, restriction of the insert from the vector and using the insert in its denatured form. This improvement was consistent whether testing serial dilutions of either whole genomic DNA or size

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3.4 kb fragment in small amounts of DNA, such as that recovered from human blood stains of 5 ul volume or human hair root tissue from individual hairs. HaeIII rather than EcoRl restriction is perhaps preferred for such samples, it giving clearer identification of the 3.4 kb fragment. Amounts as low as 5 ng of DNA have been detected using both [32S] and biotin labelled 228S probe.

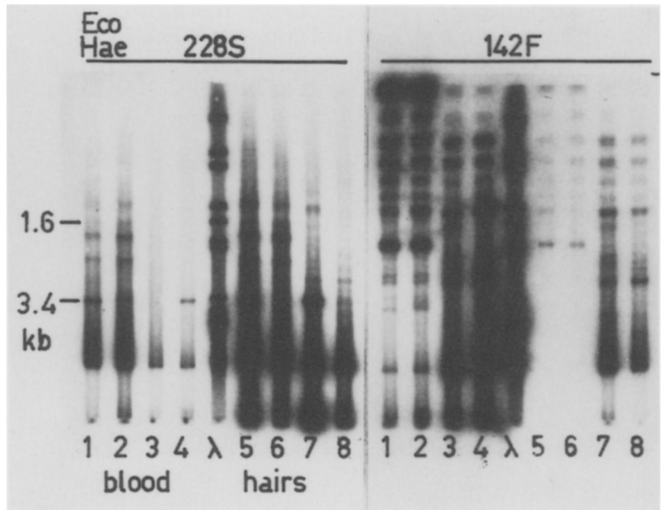

Fig. 1 EcoRl and Hae III analysis with probes 228S and 142F of blood and single hair root DNA. Samples 1,4 and 5,7 are male blood and hair DNA cleaved with Eco and Hae respectively. Samples 2,3 and 6,8 are female blood and hair DNA cleaved with Eco and Hae respectively. λ is DNA markers.

Restriction with Taq 1 and probing with 228S produced complex fragment patterns (Fig. 2). These are highly discriminating of both unrelated and related individual genomes (fig. 2). The equality of a Taq 1 restriction for each sample was also checked by stripping each membrane of sequence 228S and reprobing with 142F. As long as digestion is complete these probes provide "invariate" banding patterns for each genome.

DISCUSSION

The very dilute alkali "Southern blot" method described gives good recovery and excellent binding of single stranded DNA to nylon membranes. The membranes do not appear to physically degrade under any of the treatments used, band resolution is good and is maintained through multiple probings. The results of Reed and Mann (1985) which demonstrated quantitative

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tris, pH 7.5) for 30 minutes at 48°C. The membrane could then be reprobed. Probe removal was never absolute, as shown by long exposure of "blank" membranes on X-ray film, but was highly efficient.

RESULTS

The efficiency of various alkali "Southern" transfer procedures were initially compared by comparing the amount of "nick-translated" DNA standard molecular size fragments transferred to the membrane with that remaining in the gel. The procedures used were only semi-quantitative, but best transfer of DNA occurred when the gel was firstly acid treated, and then either blotted directly with alkali (0.4 M NaOH) (similar to Reed and Mann, 1985) or where the gel was treated with alkali (0.4 M NaOH) before blotting with substantially lower concentrations of alkali (0.02 M NaOH or less). Recovery was not as good when the gel was both treated and blotted in the higher concentration of alkali (0.4 M NaOH). Transfers at best were never complete and did not exceed approximately 70% efficiency for the range 20 kb to 0.5 kb DNA.

The same conditions were applied to the transfer of human DNA restriction fragments. Equal concentrations of either EcoRl or HaeIII restricted placental or sperm DNA were blotted to membranes and hybridised under identical conditions with probe 228S. The procedure of acid treatment, in situ alkali denaturation and then transfer using very dilute alkali (0.02M NaOH or less) provided small but consistent improvement in autoradiograph band intensity over other methods.

Comparison of the HaeIII/228S restriction patterns obtained from male DNA with female DNA (either placental or hair root), showed a 3.4 kb restriction fragment to be present in the male but absent in the female (Fig. 1). Additionally high molecular weight material unresolved in the gel and resistant to HaeIII digestion was present in both. This appeared largely to be Satellite III sequence related, there being little or no hybridisation of the alphoid satellite probe 142F in this region. Such probing showed the typical HaeIII/alphoid ladder-like banding (Fig. 1).

The EcoRl/228S restriction patterns obtained from male and female DNA again showed a means to distinguish them, though the patterns are more complex. The male tissue has an intense band at 3.4 kb and also has common banding with female DNA at 1.3, 1.7 and 2.0 kb (Fig. 1). However female DNA showed very weak banding at 3.4 and 3.5 kb, the intensity of which was found to be restriction dependent. Such results were reproducible, provided restriction was thorough, as could be monitored by reprobing with alphoid satellite probe 142F.

Either method, restriction with EcoRl or HaeIII and probing with 228S, is sufficiently sensitive to identify the male

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Southern Blotting

Gels were soaked in dilute HCl (0.25 M) for approximately 10 minutes (until the Bromophenol blue (BPB) turned from blue to yellow) and then blotted by Southern transfer to nylon membranes (Bio-Rad Zeta Probe) in dilute alkali (0.4 M NaOH) (Reed and Mann, 1985). Alternatively, after acid treatment, the gels were soaked for approximately 10 minutes in dilute alkali (0.4 M, NaOH, BPB turns from yellow to blue) and then blotted to nylon membranes using lower concentrations of alkali (0.02 M, 0.004 M NaOH). Blotting was conducted for approximately 14 hours. The membrane was then recovered and washed for 10 minutes in a neutralising solution (0.3 M NaCl, 0.03 M trisodium citrate = 2 x SSC; pH 7.0) and either stored moist in plastic bags at 4°C, or probed directly.

Probe preparation

The sequence 228S was obtained from a human sperm DNA library enriched in repetitive sequences. It has been subcloned in M-13. The sequence 142F was obtained from human fibroblast DNA.

Probes were prepared by hybridisation of an oligonucleotide primer (Biotechnology Research Enterprises, S. Australia, BRESA) to the 5' side of the insert in the M-13 vector (primer 15 ug/ul, 4 ul; M-13 with insert, 20 ul; primed in a cooling temperature gradient; 80° to 37°C for 90 minutes). Synthesis of the complementary strand using [32P]-ATP (2 ul, 1800 Ci/mmole) or [35S]-ATP, (> 1000 Ci/mmole specific activity) and CTP, GTP, TTP (2 mM each) was with the Klenow fragment of DNAase I (Klenow-BRESA 4 ul; 3 units/ul) at 37°C for 30 minutes. The whole construct was precipitated by ethanol/sodium acetate, cooled on ice, centrifuged and washed twice with 70% ethanol to removed unicorporated nucleotides. The dry residue was dissolved in approximately 3 ml of hybridisation buffer (0.7 M, NaCl, 50 mM NaH_2PO_4, 5 mM EDTA containing 1% SDS and 0.5% non-fat milk powder).

Hybridisation, washing and exposure

The membrane was routinely prehybridised for 3 hours at 68° in hybridisation buffer and then hybridised with the probe for 14 hours at 68°C using a perspex cassette. The membrane was then washed within the cassette with solutions of increasing stringency at 37°C (Wash A, 2 x SSC with 0.1% SDS, 15 minutes; Wash B, 0.5 x SSC with 0.1% SDS and trace quantities of proteinase K, 2 hours; Wash C, 0.1 x SSC with 0.1% SDS, 15 minutes; Wash D, 0.1 x SSC with 1% SDS, 15 minutes at 48°). The membrane was dried thoroughly at 38°C and exposed at ambient temperature to x-ray film (Kodak X OMat RP Film) normally with an intensifier screen (when using [32P]).

Membrane Stripping

Membranes were stripped of probes by washing twice, with agitation, in alkali (0.4 M NaOH) for 15 minutes at 48°C and

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MATERIALS AND METHODS

Isolation of DNA

Bloodstains on cloth (or single (anagen) roots for hair samples) were digested at 37°C with continuous agitation for 14 hours in an aqueous buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl, pH 8.0) containing 1% SDS and proteinase K (50 ug/ml). Digest volumes were a minimum of ten times the volume of the estimated volume of any stain. Hair root tissue was digested in 50 ul of digest buffer. The aqueous digests were extracted with 0.5 volumes phenol (redistilled, aqueous saturated pH 7.6) and once with 0.5 volumes chloroform. The DNA was precipitated by addition of 2.5 volumes redistilled ethanol and 0.1 volume sodium acetate (3 M, pH 6.0). This was stored on ice for 30 minutes and then centrifuged at 12000 rpm for 15 minutes at 4°C. The resultant pellet was washed once with 70% ethanol and dried at 37°C. Placental DNA was isolated according to Wolf et al (1980). Sperm DNA was isolated from whole human ejaculates which were allowed to liquify after collection and then the cellular component centrifuged and washed three times in an aqueous buffer (10 mM Tris, 10 mM EDTA, 100 mM NaCl; pH 8.0). The sperm pellet was demembranised by vortexing the sperm pellet in the same buffer containing 2% SDS. The resultant sperm head preparation was digested with proteinase K in SDS buffer as for bloodstains, but with the addition of 2-mercaptoethanol (0.04 M). The DNA was extracted, precipitated and washed as described above.

Restriction of DNA

The DNA recovered was restricted in minimal volumes using either EcoRl, HaeIII or Taq 1 (Boehringer Mannheim) following the manufacturers' instructions. The restriction fragments were precipitated with ethanol/sodium acetate, cooled, centrifuged, washed and dried as described by Zengin and Hartley (1985), then dissolved in 8 ul of buffer (10 mM tris, 1 mM EDTA, pH 8.0). Direct-loading of the digest into the electrophoresis gel is also possible, thus eliminating the precipitation step. This usually had no discernable effect upon the clarity of the DNA separation. One ul of a loading solution (5% Ficoll 400, 0.1% bromophenol blue (BPB), in electrophoresis buffer) was added to each 8 ul sample prior to injection into the gel.

Electrophoresis

Electrophoresis was in a 1% agarose gel (50 ml Calbiochem Type C agarose, Pharmacia GNA 100 minigel apparatus). The gel and tank buffer were identical (10 mM Tris, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.8 with acetic acid). Electrophoresis (approximately 4 V/cm) was conducted for a minimum of 2.25 hours at ambient temperature. DNA molecular size markers were included in each gel (nick translated DNA-EcoRl + HindIII, MW marker III, Boehringer Mannheim).

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A Protocol Using An Alkali Blotting Procedure For The Analysis Of Restriction Length Fragments Of Human DNA.

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ABSTRACT

The sensitivity and practicability of a combination of techniques have been investigated in a study of human tissues using restriction length fragments of DNA. In the protocol established the DNA was restricted using either EcoRl, HaeIII or Taql restriction enzymes. Studies of the capillary transfer step ("Southern blotting") of the DNA fragments from agarose gels to nylon membranes showed that the use of very dilute alkali gave improved yields. The DNA was probed with a Satellite III sequence related probe (228S) labelled with [32P]-ATP. The HaeIII/228S and the EcoRl/228S restriction patterns both show discrimination between male DNA and female DNA. The Taq 1/228S patterns are complex and have been shown to be highly discriminating of individual genomes. They have been able to distinguish between four members of a three-generation family. The protocol is satisfactory for the analysis of very small quantities of DNA.

INTRODUCTION

Capillary transfer of DNA fragments ("Southern Blotting") from electrophoresis gels to receptor membranes followed by hybridisation of the bound DNA with suitably labelled oligonucleotide probes is well established methodology (Mathew, 1984). Recently nylon membranes have been shown to bind (apparently covalently) single stranded DNA which is both denatured and transferred in dilute alkali (Reed and Mann, 1985). An advantage in forensic science of this method is that multiple probing of membranes is possible.

We have investigated the optimal conditions of alkali transfer. Such conditions should maximise the quantity of DNA transferred from the gel to the membrane and maximise the proportion which is single stranded and therefore available for hybridisation with single stranded probes.

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Table 5. Segregation of DNA fragments in a large sibship

Transmission of to Number children	Father Single fragment Obs	Father Single fragment Exp	Father Pair fragments Obs	Father Pair fragments Exp	Mother Single fragment Obs	Mother Single fragment Exp	Mother Pair fragments Obs	Mother Pair fragments Exp
0	0	0	(0)	0	0	0	(0)	0
1	0	0.1	2	1	0	0.1	0	1
2	1	0.4	3	3	2	0.4	2	3
3	3	1.3	11	11	1	1.1	14	10
4	4	3	17	25	6	2.7	16	23
5	6	4.8	38	41	4	4.3	32	38
6	5	5.6	44	44	2	5.0	41	43
7	3	4.8	43	41	4	4.3	39	38
8	2	3	33	25	2	2.7	28	23
9	1	1.3	13	11	1	1.1	11	10
10	0	0.4	5	3	0	0.4	7	3
11	0	0.1	1	1	0	0.1	0	1
12	0	0	0	0	(0)	0	0	0

(0) Homozygous and allelic bands removed for transmission analysis Transmission frequencies:

Father's fragments: 44.3%±3.1%
Mother's fragments: 44.0%±3.4%

A mutation was observed in this family (Figure 4), two further mutations have been observed in other family studies. Pooling the data gives a mutation rate of approximately 1 in 750, derived from 3 observed mutations in 2202 meioses, comparable to that of Jeffreys et al (1985). It may be postulated, in agreement with the linkage analysis above, that linkage disequilibrium is unlikely to exist in probes which have significant mutation rates. Thus the estimate of random association, even in the presence of linkage, would remain unaffected preserving the value of the test for criminal work where discrimination between individuals is paramount.

Finally it should be remembered that the Tm of RNA-DNA hybrids is significantly greater than that of DNA-DNA hybrids and, therefore, hybridisation at 65°C involving RNA probes would presumably be at 'low' stringency.

The full protocols for the use of these HVR probes are available, requests should be addressed to DJW. It is hoped that the probe 3' alpha-globin will form the basis of an international collaborative study for paternity and criminal work.

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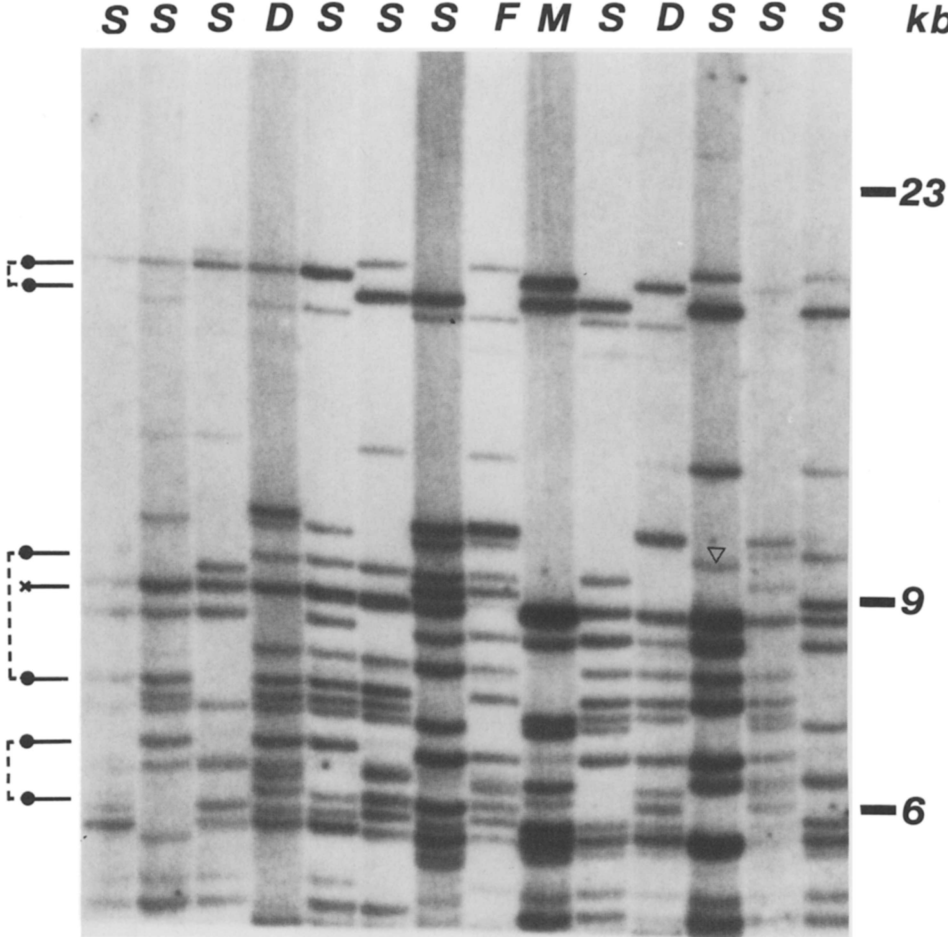

Figure 4. A sibship of 12 children showing the Mendelian inheritance of multiple DNA fragments. Allelic pairs are indicated by the dotted lines, possible homozygous loci are denoted by (*). Figure 5 is a short exposure autorad revealing fragments between 4kb and 23kb whereas this figure shows improved resolution to the 6 to 23kb range. F=Father, M=Mother, S=Son, D=Daughter,▽= Mutation to a new length allele.

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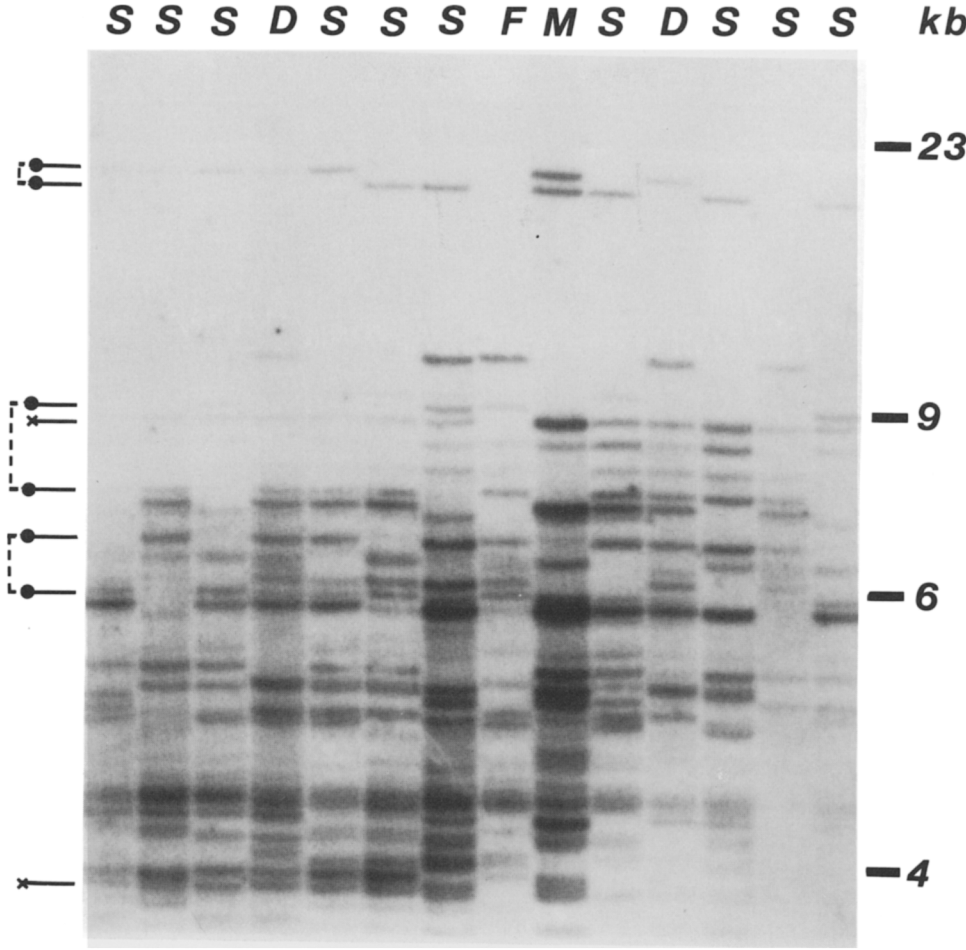

Figure 5. Short exposure autorad of a large sibship, see Figure 4 Legend.

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an average of 15 for 33.15 (Jeffreys et al 1986). The patterns produced were highly variable as reflected by the band sharing statistics and probabilities of individuality given in Table 4.

Table 4. Similarities of DNA fingerprints between random pairs of individuals for 3' alpha-globin

DNA fragments size (kb)	No. of fragments per individual (SD)	Probability that fragment A is present in B
10-23	5.25(±1.7)	0.16
6-10	6.8 (±1.92)	0.2
4-6	9.97(±1.44)	0.31

Mean probability that all fragments detected in an individual A are also present in B is given by:

$$0.16^{5.25} \times 0.2^{6.8} \times 0.31^{9.97} = 7.00 \times 10^{-15}$$

Segregation analysis

The segregation of individual DNA bands was investigated by examining the inheritance of fragments in a large sibship comprising 12 children (Figures 4 and 5). It was possible to score 25 paternal bands and 22 maternal bands using autoradiographs of different intensities. The transmission of scored bands is given in Table 5, fragments of less than 4kb were not scored.

The segregation data were analysed according to the method of Jeffreys et al (1986) in which segregations were compared against the expected binomial distribution. Within the bands there were two which were present in all of the children, as these were probably from homozygous loci they were eliminated from the transmission frequency calculations. Heterozygous paternal and maternal bands were transmitted to approximately 44% of the progeny. The number of children receiving each fragment was, therefore, consistent with a 1:1 segregation and approximated to the expected binomial distribution.

There was no evidence of linkage indicating that the DNA fragments are randomly distributed throughout the genome. However there were 3 allelic pairs. Presumably the second alleles of the remaining loci were smaller than 4kb and, therefore, not observed. The absence of linkage and the small amount of allelism would suggest that this probe can be used for paternity studies and analysis of linkage with genetic disease.

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Method

DNA was extracted as for 33.15, however for the family studies, in particular, the conditions of electrophoresis were modified: a 0.85% agarose gel was used and the samples run until the 4kb marker was 3cm from the end of the gel.

The labelled RNA transcripts of the probe to 3' HVR were produced using a commercial SP6 labelling kit (Amersham Cat. No. RPN 1506). After removal of the DNA template the probe was purified by G50 Sephadex spun column (Maniatis et al 1982), followed by phenol/chloroform extraction and alcohol precipitation in the presence of 6M ammonium acetate.

The probe was hybridised to the filters at 65°C overnight using the method of Church and Gilbert (1984). Autoradiography was carried out as for 33.15.

Chance association

DNA fingerprints from 40 unrelated individuals were examined (Figure 3). On average 22 bands were visualised in the range 4-23kb per individual, this compares favourably with

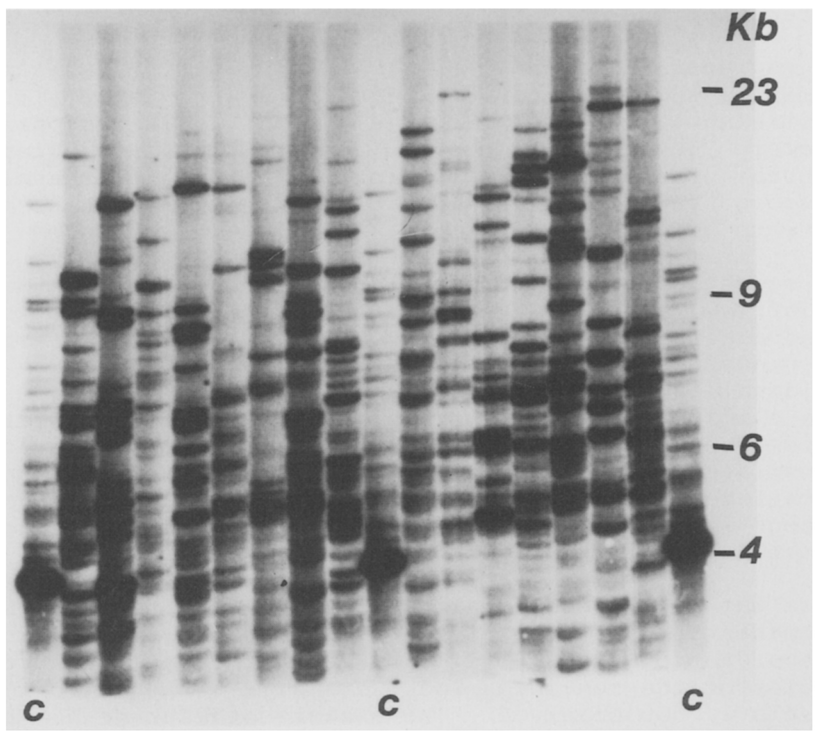

Figure 3 DNA fingerprints from 15 unrelated individuals. The control sample is denoted by (c).

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tests can then be carried out on the supernatant without jeopardising any subsequent DNA tests.

Prior to DNA fingerprinting at the Central Research Establishment tests were carried out on material from this case by Dr A J Jeffreys (Personal Communication). He used locus specific probes to exclude the suspect and suggested that the same individual could have been responsible for the 2 murders (frequency of chance association was calculated as 1 in 10,000). The advantage of using probes for the locus specific analysis of hypervariable regions is that the band pattern is uncomplicated, typically two bands. Population studies can reveal the heterozygosity of the locus and the common alleles can be readily determined. In contrast the simultaneous analysis of many highly polymorphic loci gives complex band patterns and the chance association of random individuals is made by statistical estimate.

A particularly useful role of locus specific probes could be, therefore, the screening of large numbers of samples.

ALTERNATIVE HYPERVARIABLE PROBES

The probes described by Jeffreys et al (1985b) were derived from tandemly repeated sequence within an intron of the myoglobin gene on chromosome 11. It is probable, therefore, that probes derived from other HVRs may also prove to be suitable candidates for DNA fingerprint analysis. Several HVRs are associated with the alpha-globin gene cluster on chromosome 16 (Higgs et al 1981; Proudfoot et al 1982; Goodbourn et al 1983; Jarman et al 1986). The alpha-globin 3' HVR, described by Higgs et al (1981) is a tandem repeat array of 17 base pairs, the number of repeats varies considerably between alleles. However no relationship is believed to exist between the core sequence of this HVR and the core sequence of the minisatellite probes of Jeffreys (Jarman et al 1986).

If a HVR probe to a tandem repeat array, like the 3' alpha-globin region, can be used to hybridise to autosomal loci throughout the human genome it may provide useful extra/alternative information to that derived from the Jeffreys probes. The minisatellites identified by the 3' alpha-globin probe should be largely independent of those identified by for example 33.15 (Jeffreys, 1985a) because of the difference in core sequence. Using RNA transcripts derived from this probe, DNA fingerprints have been obtained under hybridisation conditions previously described by Church and Gilbert (1984). We have examined the relationship between band patterns derived from both unrelated and related individuals.

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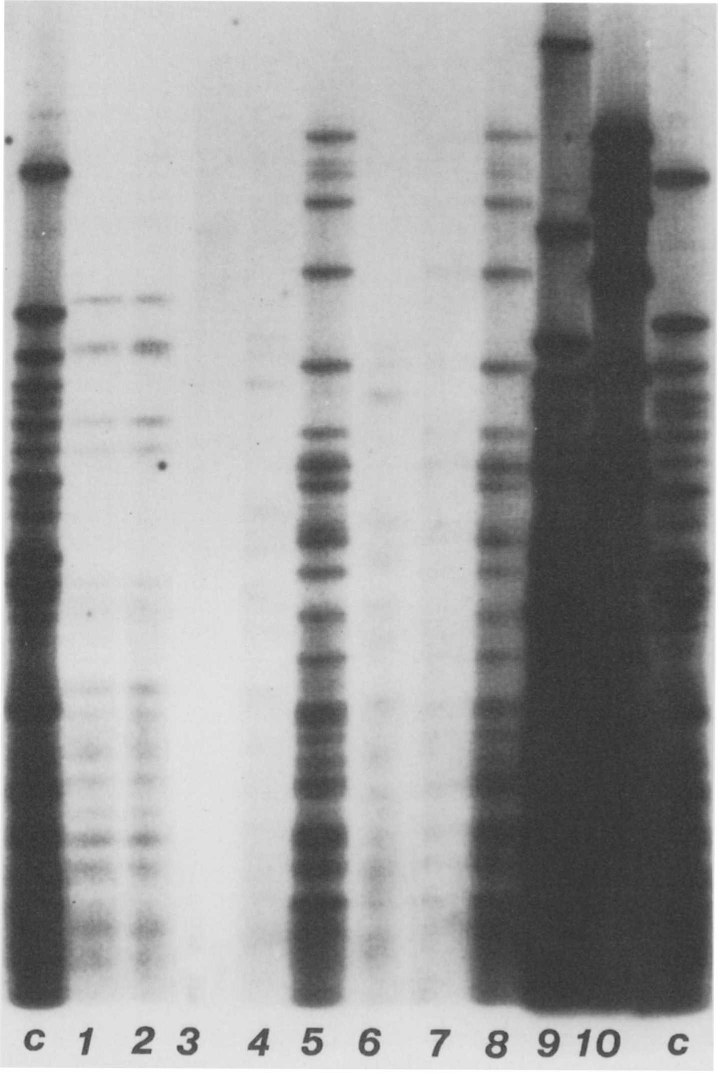

Figure 2. A case example: the association of two murders where the victims had also been sexually assaulted. The suspects fingerprints, Lanes 1 and 2, do not match the profile obtained from semen staining the pubic hair of victim 1, Lane 5, nor does it match the profiles obtained from semen staining a vaginal swab, Lane 8, and skirt, Lane 10, from victim 2. However the semen profiles obtained from the victims are identical. Note that the profile given by the blood sample from victim 2, Lane 9, demonstrates that the female contribution to the putative mixture of body fluids on the vaginal swab, Lane 8, and also perhaps the skirt, Lane 10, has been successfully eliminated.

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at the Home Office Forensic Science Laboratory, Huntingdon, Cambridgeshire, UK.)

Samples were available from both cases: these comprised semen stained pubic hair from the 1983 victim and a semen stained skirt and vaginal swabs from the 1986 victim. Blood samples/blood-stains were also available from the accused and the two victims.

Preferential extraction of the seminal DNA was carried out and, together with purified DNA from the control bloods/blood stains, electrophoresis and Southern blotting was carried out as described above. The filter was pre-hybridised in Denhardt's/carrier DNA and then probed with 33.15, produced from a guanine rich template force cloned into M13 mp8.

From the results shown in Figure 2 it can be seen that the seminal profile from the 1983 victim (Lane 5) and the 1986 victim (Lanes 7, 8 and 10) are the same, virtually confirming that they had originated from the same individual (chance association of less than 5.8×10^{-8}). However these profiles are different from the profiles obtained from the suspect, Lanes 1 and 2. Also illustrated quite clearly by the figure is the effectiveness of the preferential extraction of semen which has provided an unambiguous answer even in the presence of vaginal material (See Lane 8, vaginal swab from victim 2 and Lane 9 blood sample from victim 2). There is no evidence of partial digests, as seen by the presence of a band at the molecular exclusion point, confirming the effectiveness of the dialysis procedure prior to restriction.

The likely explanation of these findings was that one man had had intercourse with the victims shortly before, or even after death, and that this man was also the murderer. The youth, who had been held in custody was released.

The use of genetic fingerprinting in this case has prevented an ill founded and costly murder trial. However since the murders took place within the vicinity of a small community and the police had exhausted the normal avenues of investigation, a screen of all men on a voluntary basis (approximately 4000), fulfilling certain criteria established by the police, was proposed. The survey was considered possible because of the combined efficacy of conventional tests and DNA fingerprinting. The conventional tests: ABO, PGM and Lewis, could be used to eliminate approximately 86% (Asec, PGM1+) of the men, the remainder would then need to be DNA fingerprinted. The screening is now underway each sample is being analysed in duplicate, over 1000 DNA fingerprinting tests are anticipated. The results of the screen will be available later this year. It is worthy of emphasis that DNA and conventional tests on semen, in particular, are not mutally exclusive even when the amount of material available for testing is limited. An extract should be made of the seminal staining and the sperm pelleted, all conventional

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Table 3. Chance co-migration of bands in DNA fingerprints

DNA Fragment size in kb	Number of fragments per individual ± S.D.		Probability that fragment in A is present in B	
	+ carrier DNA ■	no carrier DNA □	+ carrier DNA ■	no carrier DNA □
10-20	1.3±1.0	2.1±1.2	0.07	0.16
6-10	3.8±1.3	4.2±1.6	0.2	0.28
4-6	5.9±1.3	5.3±1.4	0.26	0.27

■ As reported for 41 individuals by Gill et al (1987) using M13 sequencing primer extension in Denhardt's/carrier DNA.
□ Results for 112 individuals using oligolabelling of Ig5ls following the hybridisation protocol of Reed and Mann (1985). The mean probability that all fragments detected by probe Ig5ls in individual A are also present in individual B is given by: $0.16^{2.1} x 0.28^{4.2} x 0.27^{5.2} = 9.8x10^{-8}$, compared with $2.5x10^{-8}$ previously reported for 33.15. Mean probability of a given band in A matching in individual B=0.26, compared with 0.22 reported for 33.15 with carrier DNA.

The detection of DNA fingerprints under conditions of low stringency requires that the conditions of the test are kept constant, small changes in the protocol, particularly the temperature of hybridisation, can cause changes in the band patterns. Furthermore if the temperature of the washes after hybridisation are allowed to rise above 61°C then the probe may be stripped from many of the most informative high molecular weight fragments. Thus it is essential that before comparisons are made between different hybridisations the controls must be carefully examined for any variance in the pattern. As a further precaution the filters can be stripped and rehybridised in the same hybridisation chamber.

CASE EXAMPLE

Within the vicinity of a small community two murders had been committed. In each case the victim, a schoolgirl, had been sexually assaulted. The murders were separated by three years, the first took place in 1983 the second 1986. A youth had been charged with murder with regard to the latter case, however, the police also suspected that he was involved in the murder that took place in 1983.

Conventional blood grouping tests: ABO, PGM and Lewis, had revealed that the semen recovered from both victims could have a common origin. However the frequency of the relevant groups was such that the semen could have come from at least 10% of the male population. (These tests were carried out

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Interpretation and Comparison of 'Fingerprints'

The removal of carrier DNA from the hybridisation solution increases the number of bands observed per individual, between 4-20kb, from a mean of 11 to 11.6 (Figure 1). The increase in band number effects the individual specificity of the fingerprints (Table 3). The chance of coincidental band sharing was re-calculated using the profiles of 112 individuals with reference to the control sample as previously described by Gill et al (1987). The increased probability calculated, P=0.26 compared with P=0.22, reflects the increase in bands per individual. However the overall probability of chance association is of the same order of magnitude.

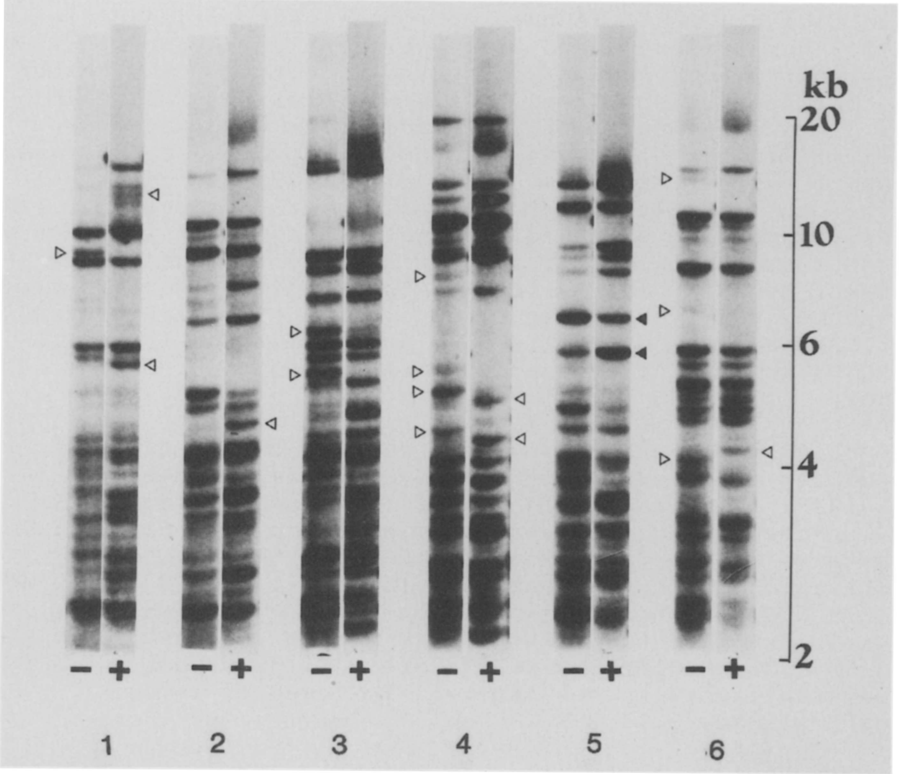

Figure 1. The effect of carrier DNA showing typical changes in banding patterns. Purified samples (1 to 6) of DNA, approximately μl g, were applied to the gel. Filters were hybridised in the absence of carrier DNA (−, hybridisation conditions after Reed and Mann (1985)). Following auto radiography the filters were stripped of probe and rehybridised in Denhardt's solution containing 0.5μg/ml carrier DNA and primer extended probe 33.15(+). Additional bands observed are denoted (▷); a reversal in intensity is denoted for 2 bands (▶).

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Electrophoresis and Blotting - Nylon Membranes: Nylon membranes offer the advantages of much greater resilience and, more importantly, after autoradiography they can be stripped of radioactivity and re-probed. Electrophoresis and Southern blotting was carried out as previously described (Gill et al 1987). Transfer of the DNA to either nitrocellulose (Schleicher and Schull, BA85) or nylon membranes (Amersham Hybond) gave equivalent results. The effect of ultra-violet irradiation on this type of nylon membrane was found to be minimal.

Probe Preparation and Hybridisation: A synthetic 400 base pair (bp) probe Ig5ls comprising 25 tandem repeats of the core sequence (AGAGGTGGGCAGGTGG) of 33.15 was used (the latter is a 592 bp probe with unique sequence DNA flanking the 29 repeats of the core sequence (Jeffreys et al 1985b)), in addition to 33.15, to develop robust, simplified protocols for hybridisation and probe preparation. Probes of high specific activity were prepared by either primer extension or random hexanucleotide priming (Amersham Multiprime Kit). Purified inserts of 33.15 and Ig5ls were used for random priming at a concentration of 0.5ng probe/ml. Primer extension probes were purified by digestion of the phage to liberate the labelled insert followed by electrophoresis in a 1.6% low melting point agarose gel. The insert was then excised from the gel after autoradiography to confirm its position. Unincorporated free radionucleotides were removed from randomly primed probes by spun columns (Maniatis et al 1982), Sephadex G-50, followed by precipitation of the DNA with 0.25 volumes of 6M ammonium acetate and 2.5 volumes of ethanol.

All hybridisation procedures were carried out at 61°C and were either as described by Jeffreys (1985a) or Reed and Mann (1985). The latter method is less complicated; dried milk is substituted for Denhardt's solution and carrier DNA. Furthermore the Reed and Mann method uses the same prehybridisation and hybridisation solutions 1.5xSSPE (1.5xSSPE is 0.27M NaCl, 15mM sodium phosphate (pH7.7), 1.5mM EDTA). Post-hybridisation the filters were washed in 3xSSC (1xSSC is 0.15M NaCl, 0.015M Sodium citrate), 0.1%SDS, until the unbound radioactivity was removed, followed by several washes in 1xSSC, 0.1% SDS.

Autoradiography and Stripping of Filters: The washed filters were covered in Saran Wrap and autoradiographed for 1-7 days with Amersham MP film and Dupont lightning plus intensifying screens at -70°C. After auto-radiography filters were routinely stripped of probe by repeated washing in 0.4M sodium hydroxide at 61°C (Gill 1987).

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The overall success rate for seminal DNA detected for all substrates was 51%. Success rate for stains was 53% (17/32) and for swabs (V.S.) 46% (6/13). The strength of the stain was estimated on a scale 0,1+H, 2+H, 3+H, 4+H. H is shorthand notation for sperm head. Chance association has been calculated using either 0.22 as the probability for band sharing (Gill et al 1987) or 0.26 depending on the hybridisation protocol (see below).

The poor correlation between assessment of sperm content, as scored on a scale 0 to 4+H, may be caused by a number of factors including storage conditions. Vaginal swabs, if not kept frozen, will create high humidity conditions within the plastic storage sleeve. DNA in bloodstains kept at high relative humidity at room temperature degrades rapidly (Gill et al 1987).

Modifications to the Method

The processing of large numbers of samples requires that the method is as simple and robust as possible. The technique has, therefore, been modified to facilitate its operational use.

Preferential Extraction of Seminal DNA from Mixtures: The preferential extraction of seminal DNA is possible because sperm heads are impervious to extraction in SDS/proteinase K/extraction buffer (0.01M Tris-HCl, 0.01M EDTA, 0.1M NaCl (pH8.0)). Female cells can, therefore, be preferentially lysed by treatment in this extraction mixture and the sperm recovered by centrifugation. However experience has shown that three washes in the extraction mixture are required, without dithiothreitol (DTT), to reliably remove the female material. The addition of DTT to the mixture causes effective lysis of the sperm head.

Dialysis and Restriction: The partial restriction of the extracted DNA, which is clearly seen by the presence of a band at the molecular exclusion point of the gel, has been eliminated by the introduction of a dialysis procedure. After the extraction and purification of the DNA (Gill et al 1985), it was reconstituted in 20μl of sterilised, distilled water and dialysed against 0.01M Tris-HCl, 0.01M NaCl pH7.6 for 2h by placing it on a floating 0.05 m Millipore filter in a 100% humidity chamber. After dialysis the DNA was recovered with a micropipette and the filter carefully washed to recover any DNA on the surface of the membrane. Restriction was carried out overnight at 37°C in approximately a 10 fold excess of *Hinf1*/core buffer made 4mM with spermidene trihydrochloride. The introduction of the dialysis procedure before restriction eliminates the partial digestion of the extracted DNA.

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Table 2. Examination of semen stained case material/swabs

Case Number	Stain Substrate	Sperm Density	Result
1	Knickers	4+H	none
	Knickers	3+H	none
2	Knickers	4+H	$5.2x10^{-4}$
3	Mac st1	4+H	$6.4x10^{-7}$
	Mac st2	4+H	$6.4x10^{-7}$
4	N.gown	2+H	$1x10^{-2}$
5	Sheet	2+H	none
	Anal swab	3+H	$5.2x10^{-4}$
6	Vag.fluid	3+H	none
7	V.S.	3+H	none
	Blouse	3+H	$9.7x10^{-4}$
8	Skirt	4+H	$5.8x10^{-8}$
	V.S.outer	2+H	$1.2x10^{-6}$
	V.S.inner	2+H	$5.8x10^{-8}$
	Pubic hair	2+H	$5.8x10^{-8}$
9	Petticoat	4+H	$2x10^{-7}$
10	Dress st1	4+H	none
	Dress st2	1+H	none
	Dress st3	4+H	$5.4x10^{-6}$
	Petticoat	2+H	none
11	Skirt	2+H	$3.8x10^{-6}$
12	Knickers	3+H	none
13	Knickers	3+H	$5.4x10^{-6}$
	V.S.lower) V.S.upper)	1+H	none
14	N.gown	2+H	none
	V.S.upper	2+H	none
	Sheet	3+H	none
	Sheet	4+H	none
15	Slip	2+H	none
16	V.S.lower	3+H	none
	V.S.mid	2+H	none
	V.S.upper	3+H	$1.1x10^{-3}$
	Knickers st1	3+H	$6x10^{-2}$
	Knickers st2	3+H	$6x10^{-2}$
	Shoe	3+H	$2.1x10^{-5}$
	Anorak	4+H	none
17	Dress st1	2+H	$8x10^{-5}$
	Dress st2	3+H	none
18	Vulval Swab	3+H	none
	V.S.outer	3+H	$5x10^{-8}$
	V.S.inner	3+H	$5x10^{-8}$
	Shirt	2+H	$5x10^{-8}$
19	Duvet cover	3+H	$2x10^{-5}$
20	Knickers	4+H	none

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The successful analysis of bloodstains requires approximately 50µl equivalent of whole blood compared with approximately 5-10µl of semen. Wide differences in success rate were found when vaginal swabs, taken at known times after intercourse, were examined from laboratory donors (Table 1).

Table 1. Semen contaminated vaginal swabs from laboratory donors

Donor	Time *post coitus*	Number of bands	Random Assoc.
1	1	10	$2.9x10^{-7}$
	3	10	$2.9x10^{-7}$
	7	none	
	8	9	$1.8x10^{-8}$
	8	none	
	13	3	$1.4x10^{-2}$
	13	none	
	18	10	$2.9x10^{-7}$
	21	none	
	21	10	$2.9x10^{-7}$
	22	10	$1.7x10^{-7}$
	24	10	$2.9x10^{-7}$
	24	10	$4.2x10^{-6}$
	25	9	$1.6x10^{6}$
	27	none	
	27	none	
	36	none	
	36	none	
	48	10	$2.9x10^{-7}$
2	2	8	$3.3x10^{-6}$
	7	8	$3.3x10^{-6}$
	11	none	
	19	8	$3.3x10^{-6}$
	22.5	4	$4.6x10^{-3}$
	32.5	4	$4.6x10^{-3}$
	42.5	4	$4.6x10^{-3}$

Chance association has been calculated for each molecular weight range: 4-6kb, 6-10kb and 10-20kb and the product of these quoted. Only the bands which could not be attributed to the female are recorded.

DNA of seminal origin was recovered on some swabs up to 48 hr after intercourse, however swabs from the same donor gave negative results just 7 hr after intercourse. The casework analyses (Table 2), which includes semen stains on textile material as well as vaginal swabs, has mirrored the wide difference in success rate achieved with the laboratory donors.

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al 1987). The ability to discriminate between individuals is potentially much greater with the latter approach, Jeffreys et al calculated that the mean probability that all DNA fragments detected in one individual by a single probe, 33.15, would be detected in a second individual chosen at random is $3x10^{-11}$. The degree of individuality achieved by locus specific probes is many orders of magnitude less. Even so the combination of selected, highly informative, locus specific RFLPs has been successfully applied to the individual identification of cell lines and, in particular, for genotypic analysis following bone marrow transplantation when recipient and donor are often closely related (Knowlton et al 1986).

Traditionally for forensic scientists using conventional test systems: ABO, PGM etc the amount of variation revealed by a single analysis may be of crucial importance, since the available material is often limited. However, novel DNA techniques allow the multiple testing of one stain extract; DNA extracted from precious case material can be examined under low and high stringency conditions using a variety of probes sequentially. Thus probes that provide an efficient and informative analysis of the genomic DNA may be selected according to the requirements of the case.

DNA fingerprinting has become an established technique at the Central Research Establishment over the past two years, it has been used for a limited number of criminal cases. These include the investigation of two associated murders which has necessitated the screening of a large number of samples. For routine operational use the technique has been simplified. Recently a probe for the HVR 3' to the alpha-globin gene has been used to produce RNA transcripts that detect many loci simultaneously. Finally the use of locus specific probes, as a useful adjunct to DNA fingerprinting, is now under investigation.

DNA FINGERPRINTING

Samples for Analysis

Sufficient DNA for several analyses can be obtained from 500µl of EDTA treated blood by high speed centrifugation. The efficient extraction of DNA from untreated samples requires the physical disruption of clots. Post mortem blood samples often yield greatly reduced amounts of high molecular weight DNA, presumably native nucleases degrade the genomic DNA as membranes rupture. Indeed because of the potential degradation of DNA by native nucleases all blood samples should be stored frozen. This may require the donor to supply two samples, one for DNA analysis and one for conventional tests, if the samples are not to be brought to the laboratory promptly.

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DNA POLYMORPHISMS - PRACTICAL USE

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INTRODUCTION

The Home Office Forensic Science laboratories in England and Wales group blood and blood-stains in the following systems: ABO, PGM, ACP, Hp and Gc. In combination these systems provide a mean discriminating power (DP) of 0.99. For semen stains the situation is much less satisfactory, only two systems are used routinely PGM and ABO. A third system, Lewis, is used to provide information to aid the interpretation of the results obtained by the ABO tests. Both ABO and PGM may give equivocal results particularly when a mixture of body fluids is present eg. semen and saliva, semen and vaginal secretion. Indeed in the examination of vaginal swabs taken after rape ABO testing will only reveal an antigen that is foreign to the victim in approximately 36% of cases (Werrett and Lang 1987). PGM tests on vaginal swabs are complicated by the stimulation of vaginal PGM production in the presence of semen, which tends to obscure the group of the seminal PGM (Garlo 1983). There is, therefore, a need for a sensitive highly discriminating test, particularly for the analysis of body fluids other than blood.

DNA ANALYSIS

Recently there have been several reports of the successful analysis of DNA extracted from both blood and semen stains (Gill et al 1985, 1987; Kanter et al 1985; Giusti et al 1985). In each case highly informative Restriction Fragment Length Polymorphisms (RFLP) have been examined which have originated from sequence re-arrangement rather than simple mutation of restriction sites. Probes which identify a particular type of sequence re-arrangement, variable numbers of tandem repeats, have been used either to identify multiple alleles at a single locus under hybridisation conditions of high stringency (Wong et al 1986; Nakamura et al 1987) or alternatively under low stringency conditions to reveal, simultaneously, alleles from many highly polymorphic loci (Jeffreys et al 1985a; Vassart et

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Detection of restriction fragment length polymorphisms can be compared to that of protein polymorphisms in so far as in each case only one locus with two or several alleles is analyzed. Therefore, in affiliation analysis cumulative probabilities have to be calculated. Investigation of minisatellites, on the contrary, in each case leads to plain yes/no decisions, i.e. identification/exclusion

basing on its extreme polymorphism in a single analysis. Because of psychological reasons, it will surely become extremely difficult to base a forensic investigation on one single test system of such potency; possibly, it will not be advisable since a single, however improbable mistake will be of such decisive significance for the further fate of an individual. In cases where conventional systems (and we include RFLP analysis as well) cannot lead to a final decision minisatellite analysis will gain substantial importance whether used in the version of Jeffreys' probes or other simple probes available to all of us.

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revealed already more than 15 bands (Figure 3). It remains to be seen whether other bacteriophages also contain such repeats which most likely have been conserved during evolution.

In the moment, the conventional procedures of gene product analysis cannot be renounced in forensic medicine. Frequencies of phenotypes are usually well known for different populations as well as linkage disequlibria for many systems. The courts are familiar with the nomenclature and the evidences. A disadvantage is found in the limited number of reproducible polymorphisms and in the relatively broad spectrum of different methods necessary for the investigations.

In paternity cases probably less than one percent of cases cannot be solved with conventional techniques. In criminological cases the percentage will be higher. So, in the long range, it will become inevitable - perhaps also more elegant - to also include the analyses of DNA polymorphisms. Their number is practically unlimited and they can all be detected using the same methodology. Presently, this system is restricted by the fact that only a few DNA probes are commercially available and that their cloning is limited to laboratories which are equipped with the necessary security facilities for gene technology. Like in the beginning of HLA analysis this only will constitute a transient inconvenience. In the backgroundt we can see already the commercial market offering a lot of kits to unexperienced investigations.

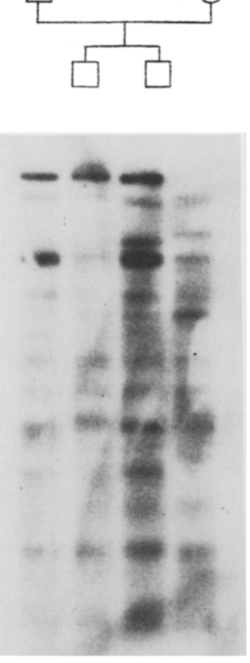

Figure 3. Autoradiogram and schematic representation of a M13-mini-satellite analysis in a family

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enormously increased when, according to Jeffreys, the repetitive core sequence used in the DNA probe is varied to some extent (e.g. probe 33.15 = 29 x 16 base pairs; probe 33.6 = 54 x 11 base pairs; see Table I). It is as yet unknown why this minor modification of the DNA sequence yields completely different patterns.

The pattern found in offspring can always be deduced from the paternal and maternal bands in a ratio of about 50:50, thus proving a strict codominant Mendelian way of inheritance of these patterns. Extensive pedigree analysis showed that some bands are simultaneously inherited indicating linkage of corresponding loci. Alternatively, the tandem repeats could be occasionally separated by short foreign sequences which then would contain a recognition site for a specific restriction enzyme. This situation explains the occurrence of "pseudo-haplotypes". The broad range of variation between individuals of the repetitive sequences at a particular locus is obviously explained by illegitimate uneven crossing over in the meiosis or by incorrect sister chromatid exchange of somatic chromosomes. The large number of alleles suggests a high mutation frequency. This fact is without relevance for the identification of persons, could, however, lead to misinterpretations in affiliation analysis. As generally known, the mutation frequency for point mutations is at about 10^{-4} to 10^{-6} per generation per locus.(This holds true also for the evaluation of RFLPs). It is estimated at about 10^{-3} for the polymorphisms of minisatellites basing on data by Jeffreys. If approximately 100 bands were analyzed in a child that would result in an a priori probability of 0.1% to find a new band, not present in the patterns of either mother or father. The probability for monozygotic twins, on the contrary, to differ by one band should be significantly lower since their separation takes place after meiosis. Presently, a precise estimation is impossible since the frequency of a somatic recombination between such repetitive sequences is unknown as yet.

The minisatellite analysis most likely will gain significant importance for forensic purposes since using a single probe will allow to evaluate a multigene family with numerous alleles. Thus, using a single test one can obtain a pattern which is specific for individuals. By now, however, the procedure is protected by patents. Even those laboratories possessing the Jeffreys' probes can solely use them for scientific purposes.

The Jeffreys probes are very interesting indeed. However, the phenomenon that already slight modifications of the core sequences result in a quite different but also reproducible fingerprint pattern encouraged many groups to work with artificial short nucleotide sequences (e.g. Epplen and coworkers, 1986) or with other natural probes. For example, Vassart et al. have demonstrated that highly specific fingerprint patterns in human genomic DNA can also be obtained by using a different, relatively simple procedure. The E. coli bacteriophage M 13, a very popular vector for cloning a whole range of various DNA sequences, contains a short sequence of 15 nucleotides (Table 1). These display no homology to the core sequences from other probes but also recognize hypervariable regions in the human genome and produce a pattern of about 40 bands. Preliminary experiments from our labo-ratory some month ago testing a family and using the whole M 13 phage DNA as probe

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The term "satellite DNA" was established because these repetitive sequences deviate in specific density from the bulk of genomic DNA by forming a separate layer in centrifugation tubes during DNA preparations in equilibrium density centrifugation.

Analysis of polymorphisms of this satellite DNA is directly connected with investigations performed by Jeffreys and his colleagues (1985, 1986). The practical steps are as follows: The DNA probe is formed by multiples of core sequences of the particular repetitive sequence (about 15 base pairs, Table 1). The genomic DNA is cut using a restriction enzyme which uses a short recognition sequence of high frequency (for example Hinf I) resulting in numerous fragments. The recognition sequence for this enzyme, however, has to be localized outside of the repeat in the flanking regions and not within the tandem repeats themselves. Otherwise two linked systems of banding patterns would be generated.

Table 1. DNA sequences of repeat units used for minisatellite probes

M13	$(GAGGGTGGXGGXTCT)_n$	core=15bp	(Vassart et al., 1987)
33.15	$(AGAGGTGGGCAGGTGG)_{29}$	core=16bp	(Jefferys et al., 1986)
33.6	$(AGGGCTGGAGG)_{54}$	core=11bp	(Jeffreys et al., 1986)

Given the conditions mentioned above, a set of fragments of varying sizes is produced. They migrate differently and form different bands which, however, can be detected using the same DNA probe. Jeffreys estimates 3 to 29 repeats as a variation range for his system of one core sequence. This would correspond to about 25 electrophoretically distinguishable alleles. When the individual number of repetitive sequences is evenly distributed over a population it would mean an average frequency of alleles of p = 0,04 and lead to the fact that practically all alleles are present in a heterozygous form ($h=1-25x(0.04)^2=0,96$). Consequently, in 96 % of the individuals two bands per locus would be recognized and located at different positions of the gel. For this system, Jeffreys coined the name "fingerprints" describing a pattern of multiple bands as shown in several publications. This complex pattern is caused by an additional polygenic situation. These repetetive sequences which can be demonstrated using one particular DNA probe occur at multiple loci in the human genome, their number corresponding to about half of the visible bands. According to the results obtained by Jeffreys one can expect 60 loci leading to a pattern of about 120 bands in each individual. For forensic purposes, this pattern is limited mainly to the fragments of higher molecular weight in the upper part of the gel containing an increased number of repeats.

In contrast to the RFLP analysis, using a set of several restriction enzymes would not yield new information (if we disregard possible polymorphisms in the flanking regions) but only a modified banding pattern. The information, however, can be

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Minisatellites

Analysis of minisatellites is aimed at the enormous polymorphism of the middle and high repetetive DNA sequences. In contrast to the RFLP analysis we are confronted here with quantitative and not with qualitative polymorphism. The biological significance of the repetive sequences is presently not understood. It is speculated that they are somehow connected with gene protection ("body-guard-hypothesis"), stabilization of chromatin, gene regulation, and possibly spontaneous DNA recombination.

For forensic analysis it is decisive that these minisatellite sequences are scattered in tandem repeats throughout the entire genome and that the copy number varies substantially in different individuals. Futhermore it is important, that these clusters are

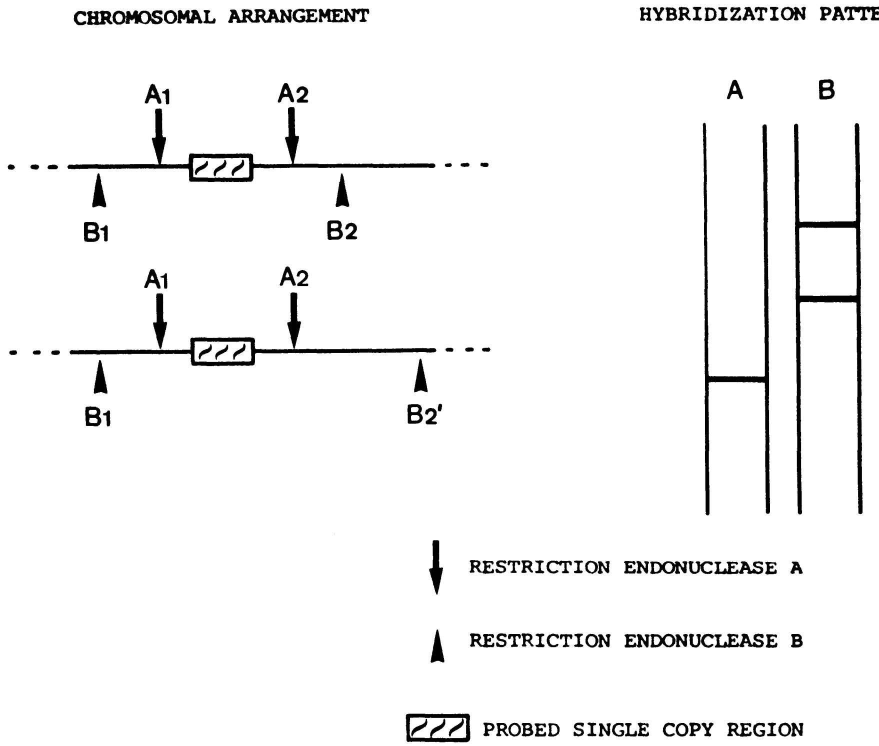

Figure 2. Schematic representation of two homologous chromosomes restricted by two endonucleases, A and B, respectively, yielding two corresponding fragments of identical size for A. B generates two larger fragmens of different size due to a deviating restriction site on one of the chromosomes (B2 and B2', respectively)

localized at corresponding loci of the homologous chromosomes. Thus, they present a form of alleles which do not, however, differ in their structure but in their length i.e. in the number of short core sequences.

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The technical steps are the following:

After electrophoretic separation the DNA fragments in the gel are denatured by alkali resulting in single-stranded DNA. Then, they are transferred from the gel to a solid support membrane (nitrocellulose or nylon). These steps constitute the classical "Southern blot" procedure. By incubating this membrane with a radioactively labeled probe a molecular hybrization occurs precisely at that position where the probe detects a homologous, complementary DNA sequence.

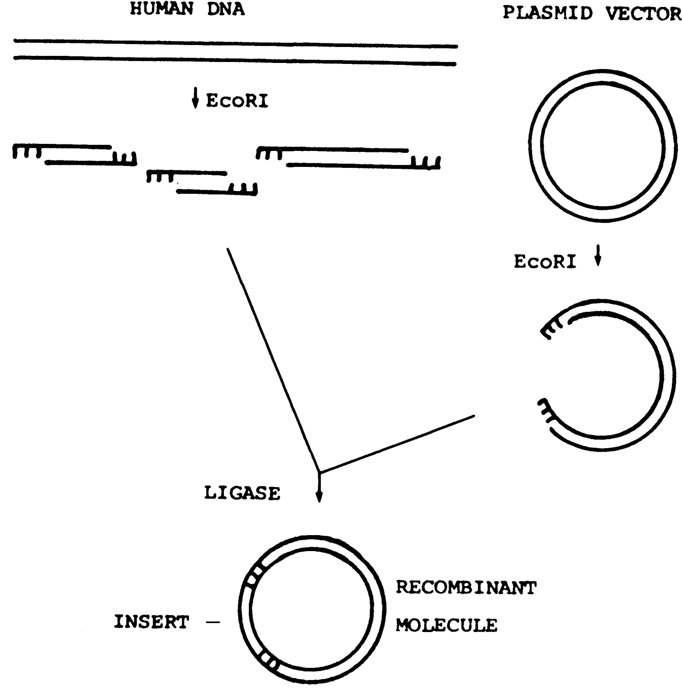

Figure 1. Double-stranded linear and circular DNA is dissected by using the EcoRI restriction endonuclease and generating "sticky ends". Then, the fragments are linked using ligase to form a recombinant DNA.

Finally, the specific bands and the resulting pattern are visualized by autoradiography (Figure 2). Since the filter can be washed after the autoradiographic step and successively incubated with different probes it is possible to search for several polymorphisms using the same gel. In the meantime, non-radioactive techniques have been developed which also show a high resolution. Under optimal conditions 20 micrograms of high molecular weight DNA can be isolated from one milliliter of blood. About 5 micrograms of DNA are necessary for gel electrophoresis; thus, DNA from one ml of blood can be studied using four different restrictions enzymes and four electrophoretic separations which then can be hybridized with a panel of probes and will result in a quadruplicate of information.

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foreign DNA, for example from penetrating bacteriophages. The application of these enzymes to human genomic DNA results in reproducible fragments of defined length which can be separated electrophoretically (for example using agarose gels) according to their molecular weight and thus to their length.

Mutations may lead to deletions, insertions or exchange of one or several nucleotides. These events can generate a new restriction site or the previously present site may disappear. This may change the size of the respective DNA fragments resulting in a different electrophoretic mobility of the bands. If this mutation presents no disadvantage for the carrier (e.g. by inactivating an important gene) it can be passed on to the next generation via germ line and can be demonstrated as a codominant polymorphism at the DNA level. As mentioned above, such restriction fragment lenght polymorphisms are rarely detected in active genes themselves. In close neighbourhood to a particular gene or in non-expressed intron sequences of a gene, however, they offer useful information for detection of patients or predisposed individuals in the area of clinical genetics by means of linkage analysis. For such an indirect analysis of the phenotype of all monogenic characteristics about 1.600 precisely localized polymorphic markers spread evenly throughout the human genome would suffice (Botstein et al., 1980). This is a number which will be reached shortly. For forensic means, the location of the markers is irrelevant and the number could be much smaller. In analogy to analysis of polymorphic proteins it is the number and relative frequency of alleles which is decisive.

DNA probes

If the complete genomic DNA of an individual were used in a restriction enzyme assay hundreds of thousands of fragments would be distributed across the gel according to their length and disguise a clear pattern, even more so a change of such a pattern. To reach this goal it is necessary to apply DNA probes. Such probes are presented by defined DNA sequences, even defined genes under certain conditions, which are amplified by cloning and radioactively labeled. Specific or anonymous DNA sequences of the human genome can be incorporated into a naturally occuring or a artificially constructed vector molecule (e.g. plasmid or bacteriophage). Dissection of the genomic DNA and of the vector molecule using the same restriction enzyme provides identical DNA termini and facilitates the incorporation of a human DNA sequence into the vector and the recircularization of the recombinant. This recombinant can be manipulated into host bacteria and thus, multiplied ad libitum (Figure 1).

This process of multiplying human DNA sequences into large copy numbers is called molecular cloning. Following the separation of the insert from the vector and tagging the DNA by a radioactive label (e.g. by "nick-translation") such DNA sequences may be used as a genetic probe in order to visualize particular genomic fragments by means of autoradiography (for technical details, see Maniatis et al., 1982). This procedure favourably applies such probes which are present in the human genome only once (single copy sequences), therefore, a simple and clear banding pattern can be obtained, as it is known from analyzing protein polymorphisms.

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Admittedly, this still represents quite a large number. Recombinant gene technology, however, allows to demonstrate all changes in the primary gene sequence, even those which occur in the 95 % of non-transcribed DNA.
Only in the minority of the cases, in particular when investigating genetic defects, we are trying to detect the (pathological) polymorphism of the gene product at the level of the gene itself. In the majority of these cases, anonymous DNA sequences which are dissected from the genome by restriction enzymes (see below) are used in recombinant gene technology. It was found that about each 1/100 to 1/200 base pair (corresponding to 0.5 - 0.1% of nucleotides) is changed by mutations thus generating a special class of "alleles" which are inherited as codominant polymorphisms at the DNA level and are present in a homozygous or heterozygous state depending on the molecular phenotype of the individual. At the level of gene product this allelism can generally not be recognized.

About 70% of our genome is represented by so called single copy genes. 20% are known as middle repetitive sequences (several thousand copies), 10% as highly repetitive sequences (several hundred thousand and more). Excluding several gene families, e. g. the ribosomal genes, leaves the repetitive DNA predominantly existing as short sequences ("minisatellites", see below). These can be found at multiple homologous loci of the corresponding sister chromosomes, for example in the centromeric region in a fashion of tandem repeats. They display an enormous individual variability in the number of copies and therefore present multiple alleles in an electrophoretic pattern specific for individuals. Point mutations and mutations of the number of repeats together result in a tremendous number of about 30 million polymorphisms at the DNA level which obviously seem difficult to relate to our biological existence. It was shown, however, that this high variability accumulated in the course of millions of years, mainly in the non-transcribed, i.e. genetically inactive DNA sequences - this means in sections of the genome which do not present a disadvantage in selection for the carrier. Analysis of these two different kinds of polymorphisms in human DNA presently results in two basically different approaches:
- the RFLP-analysis, i.e. detection and evaluation of the restriction fragment length polymorphisms which mainly represent a Mendelian two or few-alleles system;
- the analysis of the minisatellite sequences i.e. detection and evaluation of hypervariable regions which correspond to a multiallele system and deliver an individual-specific DNA pattern.

RFLPs

Using bacterial restriction endonucleases high molecular weight human DNA can be dissected into numerous fragments. These enzymes are distinguished by the fact that they do not cut the DNA unspecifically resulting in ever shorter oligonucleotides in the course of their action but that they only act in very specific recognition sequences or at a defined distance from this recognition sites. These recognition sites are composed of 4 to 8 base pairs. Presently, more than 200 of such enzymes catalyzing the specific section of double-stranded DNA are known. Their biological significance obviously lies in protecting bacteria from

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RECOMBINANT DNA TECHNOLOGY AND HUMAN DNA-POLYMORPHISM

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Hitherto, only phenotypical markers from blood, other tissues and excretory fluids served in the process of identification of persons from stains and in affilitation analysis. They base on two phenomena:

- numerous human characteristics are monogenically inherited and display polymorphisms at the level of the gene product, i.e. they are not homogeneous;
- these polymorphisms strictly follow Mendelian laws of dominance and recessivity. Generally, they are inherited in a codominant way, in exceptions in a dominant/recessive way (e.g. ABO system).

The occurrence of a variant at a frequency of at least one percent is called polymorphism; in the codominant way of inheritance this corresponds directly to its gene frequency. The empirical value of 1% guarantees a population genetic equilibrium. Variants with a lower frequency are essentially determined by new mutations. This is useful for the identification of persons but may cause substantial problems in paternity testing.

In paternity cases the routinely applied set of about 20-30 systems (ABO, MNSs, P, Rh, K, Fy, Jk, Lu, Xg, Se, acP, AK, ADA, PGM1, GPT, EsD, GLO, HP, Gm, Km, Gc, C3, Bf, Tf, Pi und HLA A, B, C) already provides a cumulative a priori-probability of exclusion of 99.9 % and a plausibility of paternity in the same range according to Essen-Möller.

Disadvantages of these systems result from limited sample size from using quite different techniques, from the uneven distribution of the corresponding genes on the chromosomes and from linkage disequilibiria (e.g. HLA genes) which also have to be taken into account. In general, this set of standard polymorphisms is sufficient for forensic application. If neccessary, it may be extended to about 40 scientifically proven polymorphic systems.

The investigation at the level of the gene product, i.e. the protein, mirrors only vaguely the extent of the polymorphisms at the level of the gene. The diploid human genome is composed of about $2x3x10^9$ nucleotides (base pairs). Considering that three nucleotides code for one amino acid and that an average polypeptide chain shows a length of 150 to 350 amino acids a number of about $3x10^6$ primary gene products can theoretically be calculated. According to realistic estimates, however, only 20.000 to 100.000 genes exist or are expressed in man. This means that we are using less than 5 % of our genetic material translating it into gene products. Out of these, about one third demonstrate polymorphisms.

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IV. DNA Polymorphisms

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Table 4 MN typing of blood stains using monoclonal reagents

Stain	anti-M diluted 1/8	anti-N diluted 1/8
MM	4+	-
MM	4+	-
MN	4+	4+
MN	4+	4+
NN	-	4+
NN	-	4+

Titre of original anti-M with MN cells = 128

Titre of original anti-N with MN cells - 64

Table 5 Example results from tests for the D typing of bloodstains using monoclonal anti-D UCH D4

Rh type of stain	Age of stain	Dilution of anti-D 1/8	1/16	1/32	1/64
CCDee	1 week	4+	2+	4+	4+
CcDEe	1 week	4+	4+	4+	4+
ccddee	1 week	2+	-	1+	-
ccddee	1 week	2+	-	-	-
CCDee	4 mths	4+	4+	4+	3+
ccdEE	4 mths	4+	4+	4+	4+
CcDEe	4 mths	4+	4+	4+	4+
ccddee	4 mths	2+	2+	1+	-

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Table 2 Tests with anti-A reagents in 3 year old bloodstains

	5138*	3D3**	6D4**	Seraclone**
A_1	C C	V C	C C	C C
A_1	C C	++ C	C C	C C
A_2	C C	- ++	+ V	C C
A_2	C C	W ++	+ V	C C
A_2	C C	- +	- -	V C
B	- -	- -	- -	- -
O	- -	- -	- -	- -

* polyclonal
** monoclonal

see also Key in Table 1

Table 3 Example results using 3 year old bloodstains with variOus anti-A and anti-B reagents

	anti-A				anti-B			
	3D3*	6D4*	Seraclone*	513**	5B2*	5A5*	Seraclone*	55441**
A_1	V C	V C	C C	C C	- -	- -	- -	- -
A_2	- ++	+ ++	C C	C C	- -	- -	- -	- -
A_2	W +	- -	++ ++	C C	- -	- -	- -	- -
B	- -	- -	- -	- -	C C	C C	C C	C C
O	- -	- -	- -	- -	- -	- -	- -	- -
A_2B	+ C	+ C	V C	C V	C C	C C	C C	C C
A_2B	- -	- -	- W	- W	C C	V C	C C	C C
A_2B	- -	- -	- ++	- V	V C	C C	C C	C C

* monoclonal reagent
** polyclonal reagent

see also Key in Tabe 1

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of the reagent have been used for the detection of the D antigen in bloodstains. Success could be achieved using a carefully selected concentration. Higher concentrations of the anti-D produced positive reactions with the D negative stains as well as with the D positive stains.

It is interesting that this reagent is completely specific when used by a variety of techniques for normal agglutination tests on fresh blood samples, thus illustrating the need for careful re-standardisation of reagents before use by elution.

Monoclonal reagents are now available and hopefully will provide exciting potential for forensic serologists but we must get to know them well before we can rely on them and be confident in their use in casework. In spite of their high degree of specificity they must be carefully standardised and selected by the technique by which they are to be used.

Table 1 Example results of elution tests using various dilutions of anti-A 3D3

	Dilutions of anti-A 3D3						
Bloodstain	Neat	½	¼	1/16	1/64	1/128	1/256
A_1	C C	C C	C C	V V	+ ++	+ ++	+ ++
A_2	V C	C C	V C	++ ++	+ ++	- ++	- +
B	- -	- -	- -	- -	- -	- -	- -
O	- -	- -	- -	- -	- -	- -	- -

C = complete agglutination
V = visual agglutination
++ = strong microscopic agglutination
\+ = medium microscopic agglutination
(+) = weak microscopic agglutination
\- = no agglutination

Two results = first reading after 30 minutes incubation with indicator cells, second reading after 2 hours incubation.

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reactions were obtained even using the undiluted reagent.

A dilution of 1/4 was selected for further investigation. A dilution of 1/4 or 1/8 was selected for most ABO reagents used in this survey. This is the same level of dilution as normally used for polyclonal reagents.

Similar succcessful results were obtained using monoclonal anti-A 6D4 from Dr Voak and a seraclone reagent supplied by Biotest Ltd., when the stains ranged in age from 1 to 12 months. Results obtained using A_1 and A_2 bloodstains which were 3 years old indicated that not all monoclonal reagents would be equally suitable for detecting weaker A activity. Example results in Table 2 show how some reagents are less efficient for detecting the A antigen in stains of group A_2.

A critical factor for judging the suitability of anti-A reagents for normal grouping of fresh blood samples is their ability to detect samples of A_2B, therefore the various reagents were used to test A_2B bloodstains. Also the stains of various ages were used as an additional method of introducing stains with different antigen strength. The AB bloodstains were also tested with anti-B monoclonal reagents which had been shown to produce satisfactory results with fresh bloodstains of groups A, B and O.

Although most of the fresher stains could be successfully grouped using the monoclonal reagents, differences in suitability became particularly marked using A_2B stains which were 3 years old. Example results are shown in Table 3. It is interesting that all the monoclonal anti-B reagents used, satisfactorily detected the B antigen, so that the possibility of typing stains as group B which were really AB could be a problem.

It appears that some workers have found monoclonal ABO reagents totally unsatisfactory in their elution tests, particularly it seems the reagents offered by Ortho Diagnostics. Results obtained using three batches of Ortho anti-A and several examples of their anti-B reagents donated to us by workers at the FBI Research Centre in Quantico, produced satisfactory grouping reactions with bloodstains of groups A_1, A_2 and B and only with 3 year old A_2B stains did we clearly fail to produce satisfactory positives with these monoclonal reagents and clear cut positives with polyclonal reagents.

Table 4 shows example results obtained using monoclonal anti-M and anti-N reagents, Seraclone, supplied by Biotest Ltd. The difficulties experienced in the past with MN typing of blood-stains means that this systems seldom find itself a place in the repertoire of forensic serologists. It is interesting that the few results we have suggest that these reagents could produce far more satisfactory MN grouping of bloodstains than rabbit antisera of lectins.

Table 5 shows example results obtained using a monoclonal IgG1 anti-D produced by Dr Crawford in London. Various concentrations

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The Use of Monoclonal Antibodies for the Detection of Red Cell Antigens in Stain Material

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INTRODUCTION

Where the grouping of stains for red cell antigens is used as a routine procedure in casework, considerable quantities of antisera are required and the availability of monoclonal reagents is a welcome development. Moreover the serologist may soon find that the most readily available reagents for our presently used techniques of inhibition and elution will be monoclonal antibodies.

Our previous experience with polyclonal reagents has shown that some antibodies are more suited than others for the detection of antigens in stains by elution and careful selection of the antisera has always been considered by us to be an essential preliminary to successful grouping. We considered monoclonal reagents could show variation in their suitability and in this study we have investigated the elution technique using mostly anti-A and anti-B reagents but also including tests with anti-M, anti-N and anti-D.

The elution technique used has been described elsewhere (Dodd and Lincoln 1982).

RESULTS

Preliminary experiments were made with a series of doubling dilutions of each monoclonal reagent to select a possible suitable dilution for successful grouping of bloodstains.

Example results, shown in Table 1 were obtained using a number of dilutions of a monoclonal anti-A, 3D3, kindly supplied to us by Dr D Voak of Cambridge. This reagent was considered to reach standards expected of normal anti-A licensed reagents. At a dilution of 1/16 and more slightly less strong reactions were obtained with the A_2 than A_1 bloodstains which were less than one month old. Also using the higher dilutions A_1 bloodstains produced stronger reactions than A_2 stains after the shorter incubation period. Positive reactions were obtained even when the reagent was diluted to 1/256. No false positive

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The suitability of the 2 MAb for Ag penotyping in serum was investigated by ELISA inhibition. A panel of 38 sera of unrelated individuals was characterized. The distribution pattern of apo B concentrations required for 50% inhibition is shown in Fig. 1 together with the relevant Ag phenotypes as determined by passive hemagglutination inhibition using human antisera. The ELISA inhibitions show 2 different patterns: on the one hand, a monophasic pattern resembling the standard distribution for MAb F5D5, indicating that this MAb does not recognize a polymorphism on LDL. On the other hand, a biphasic pattern for Mab D2E1 and H11G3 is typical for the recognition of a polymorphism on LDL. D2E1 was strongly inhitited (<240 µg apo B/ml at 50% inhibition) with homo- and heterozygous sera containing Ag epitope c (Ag c+/g- and Ag c+/g+), while inhibition with homozygous sera containing only the allelic epitope g (Ag c+/g+) was weak (>320 µg apo B/ml). H11G3 was strongly inhibited (<480 µg apo B/ml at 50% inhibition) with homo- and heterozygous sera containing Ag d (Ag a1-/d+ and Ag a1+/d+) and weakly (>800 µg apo B/ml) with homozygous sera lacking this epitope (Ag a1+/d-). Bi- or triphasic patterns were also reported with other Ag c specific MAb in various assay systems (Robinson et al. 1986, Tikkanen et al. 1986, Young et al. 1986 and Duriez et al. 1987).

So far, only Ag c specific MAb have been described in the literature. If - in addition to the anti-Ag d specific H11G3 - MAb against further Ag epitopes can be produced, it should be possible to replace the human antisera in Ag phenotyping. Thus, the problems imposed by the shortage of these antisera could be overcome.

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as binding to coated LDL 39 was strongly inhibited by LDL 39 and 405, the only two LDL bearing this epitope. H11G3 recognizes Ag d, since strong inhibition was observed with LDL 39, 21, 209 and 405, the 4 LDL bearing this epitope. F5D5 was inhibitable by all 6 LDL as this MAb recognizes a common epitope on apo B.

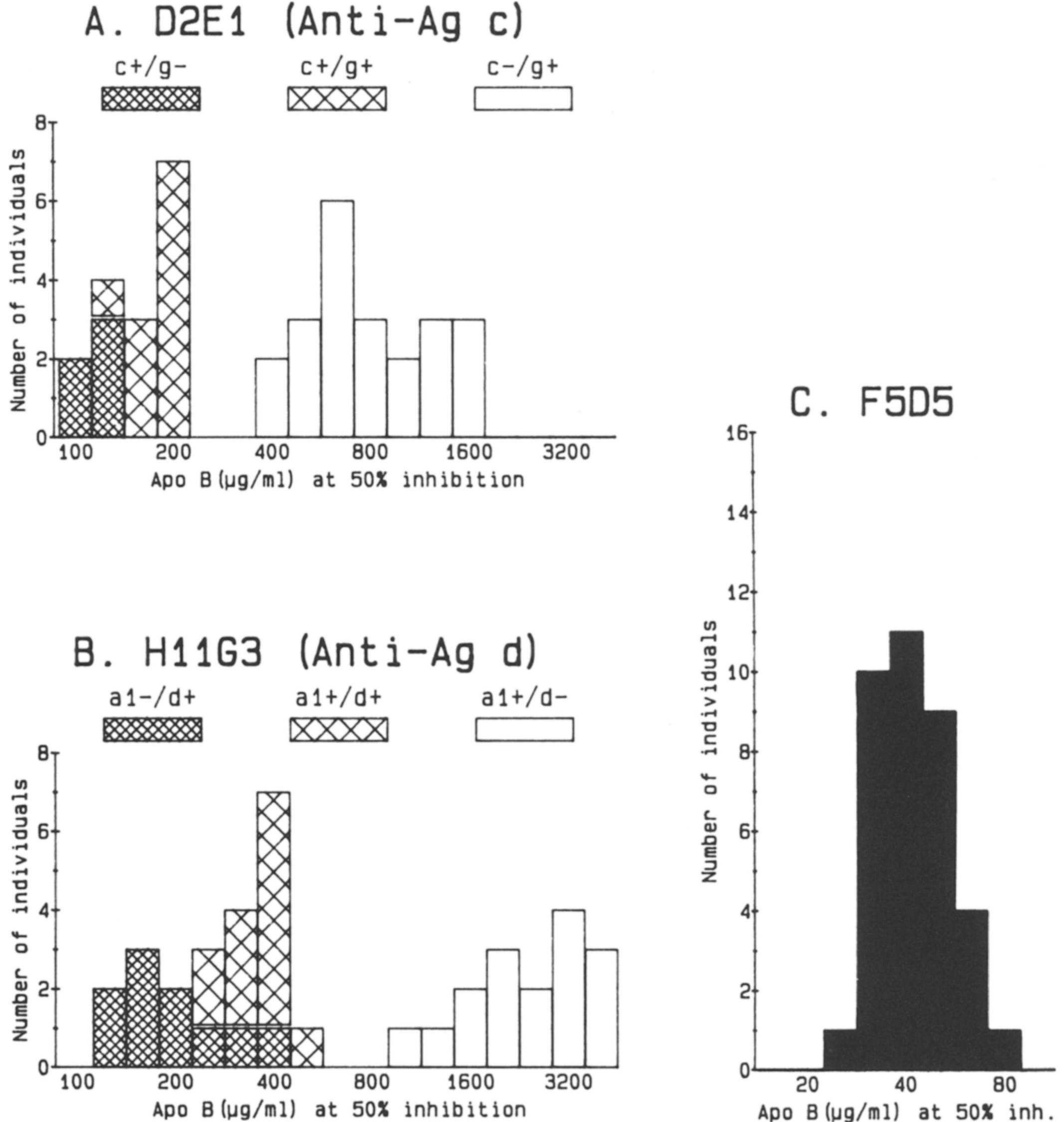

Figure 1. Reactivity of MAb with a panel of 38 sera from unrelated individuals in ELISA inhibition. ELISA inhibition was performed as described in the methods. The results are expressed as apo B concentration at 50% inhibition.

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Titertek Multiscan MS automated reader. To determine the apo B content in serum, the MAb was replaced by a polyclonal rabbit anti-human apo B antiserum. These plates were washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG followed by incubation with substrate.

RESULTS AND DISCUSSION

Hybridoma cultures were screened as described in the methods. Two MAb - D2E1 and H11G3 - were selected and further cloned by limiting dilution. MAb F5D5 recognizes a common epitope on LDL and was used as a control.

Table 1. Ag phenotypes of individual LDL with respect to the 5 epitopes expressed on the immunogen LDL 39 and determination of the specificity of MAb D2E1, H11G3 and F5D5 by ELISA inhibition

LDL	Ag epitopes[a]					μg apo B/ml at 50% inhibition			+/- evaluation[b]		
	y	d	c	t	i	D2E1	H11G3	F5D5	D2E1	H11G3	F5D5
39	+	+	+	+	+	71	172	37	+	+	+
21	+	+	-	+	+	537	62	43	-	+	+
24	+	-	-	-	+	327	407	30	-	-	+
T168	-	-	-	+	+	457	631	29	-	-	+
209	-	+	-	+	+	468	100	25	-	+	+
405	+	+	+	+	-	61	37	25	+	+	+
Recognized epitope:									c	d	common

[a] LDL 39, 21, 24, T168 and 405 are homozygous with respect to all 5 pairs. LDL 209 is heterozygous with respect to the pair a1/d and homozygous with respect to the other 4 pairs

[b] + for strong inhibition (<200 μg apo B/ml at 50% inhibition) and - for weak inhibition (>300 μg apo B/ml)

The specificity of the 2 selected MAb was investigated by ELISA inhibition with 6 different LDL preparations previously phenotyped with human antisera by passive hemagglutination inhibition (Table 1). The values for the apo B concentrations at 50% inhibition were determined graphically from the inhibition curves and submitted to +/- evaluation (Table 1). According to the Ag phenotypes of these 6 LDL, D2E1 recognizes Ag epitope c,

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Monoclonal Antibodies Specific for Ag c/g and Ag a1/d Polymorphism of Human Low Density Lipoprotein

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INTRODUCTION

Human low density lipoprotein (LDL) is the major cholesterol carrier in the blood stream. It occurs as spherical particles and contains one large protein molecule, apolipoprotein B (apo B).

A genetic polymorphism of this protein was discovered in 1961 by Allison and Blumberg. Subsequently, 5 pairs of Ag epitopes behaving as products of allelic genes were defined using human allotypic antisera: x/y, a1/d, c/g, t/z, and h/i (Bütler and Brunner 1974). The determination of these epitopes has become a usefull tool in paternity investigations, forensic medicine, twin diagnosis, family- and population genetic studies.

The scarcity of human allotypic antisera poses a problem because relatively few polytransfused patients produce anti-Ag antibodies and transfusion of whole blood is replaced more and more by transfusion of blood components lacking allotypic LDL. Therefore, we produced monoclonal antibodies (MAb) against Ag epitopes and obtained anti-Ag c and d in a first attempt.

MATERIALS AND METHODS

LDL was prepared by ultracentrifugation and Ag phenotyping of sera and LDL preparations was performed by passive hemagglutination inhibition using human allotypic antisera (Bütler et al. 1967). Apo B concentrations were determined by radial immunodiffusion.

Female Balb/c mice were immunized with LDL of phenotype y+x-a1-d+c+g-t+z-h-i+ (LDL 39). The spleen cells were fused with X63-Ag8.653 mouse myeloma cells using polyethylenglycol 4,000 d. Pooled supernatants from 2 days of HAT-selected hybridoma cultures were assayed by ELISA for antibodies binding to LDL of different Ag phenotype (LDL 39, 24, T168 and 405; see Table 1). Hybridomas which produced antibodies to LDL 39 but not to at least one of the other LDL were cloned by limiting dilution. MAb were produced in ascites, purified on a Bakerbond Mab 4.6 x 250 mm HPLC column and biotinylated with biotin-X-NHS (biotinyl-ε-aminocaproic acid N-hydroxy succinimide ester).

For ELISA inhibition, flat-bottomed microtitration plates were coated with LDL 39 and remaining free sites blocked with casein hydrolysate. The plates were incubated with serum or LDL in 2-fold dilutions together with a constant amount of biotinylated MAb. The plates were washed and incubated with alkaline phosphatase-conjugated streptavidin and developed with p-nitrophenylphosphate. The absorbance was measured at 405 nm in a

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Table 3. IgM monoclonal anti-D: reactivity with various D phenotypes

Antibody	lc	D category IVa	IVb	Va		VI	VII		Rh:33		Titre against CDe/cde cells		
		s	s	s	p	p	s	p	s	p	s	p	IAT
FOM A	λ	+	+	+	+	-	+	+	$+^w$	+	4	4	1
FOM 1	λ	+	+	-	-	-	+	+	-	-	16	16	4
HAM A	k	+	+	-	-	-	$+^w$	+	-	+	16	64	4
HAM B	λ	+	+	-	-	-	-	+	-	-	64	128	8
HAM 1		-	-	-	$+^w$	-	+	+	-	-	8	8	8
HAM 2	λ	+	+	+/-	+	-	+	+	-	+	64	128	8
MAD		+	+	-	$+^w$	w/-	+	+	-	w	>512	> 512	64
MAD 1	λ	+	+	-	+/-	-	$+^w$	+	-	-	a	a	a
MAD 2	λ	+	+	-	$+^w$	-	$+^w$	+	-	-	128	128	8
HD7	k	-	-	+	+	+	+	+	-	-	2	4	2

Category IIIa, IIIc gave strong positive results by all methods against all antibodies.

lc = Immunoglobulin light chain type.
s = results of agglutination tests using 3% saline suspension of untreated cells.
p = results of agglutination tests using 3% saline suspension of papain treated cells.
IAT = Indirect antiglobulin test using polyspecific anti-human globulin and untreated cells
a = insufficient reagent available for titration.

Strength of reaction order +, $+^w$, W, -
+/-, w/- = positive or weakly positive with some samples, negative with others.

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Table 1. Classification of people with D on their cells who have made anti-D

	anti-D from D+ people						Anti-		
cells	II	IIIa	IVa	Va	VI	VII	Go^a	D^w	Tar
D II	-	+	+	+	+	nt	-	-	-
D III	+	-	+	+	+	w	-	-	-
D IV	-	w	-	+	+	+	+	-	-
D V	+	-	+	-	±	-	-	+	-
D VI	-	-	+	-	-	-	-	-	-
D VII	+	-	+	±	+	-	-	-	+

nt not tested

Table 2. IgG monoclonal anti-D: reactivity with various D phenotypes

Antibody	Ig		D category					Titre against CDe/cde cells	
	sc	lc	IVa	IVb	Va	VI	Rh:33	p	IAT
FOG A	G3	k	+	+	+/-	-	-	32	8
FOG B	G1	λ	+	+	+	-	-	256	256
FOG C	G3	k	+	+	+/-	-	-	64	32
FOG 1	G1	k	+	+	+	-	w	64	32
FOG 3	G3	k	+	+	+/-	-	-	128	32
PAG 1	G1	λ	+	+	+/-	-	w	64	16
REG A	G1	k	-	-	-	-	-	256	32
8D6	G1		w/-	w/-	-	-	-	32	2
GAD	G3		+	+	-	-	-	64	32
GAD 2	G3	λ	w	w	-	-	-	64	32
UCH D4	G1	k	+	-	+	w/-	-	>512	16

Category IIIa, IIIc and VII gave strong positive results against all antibodies.

Papain treated cells (3% saline suspension) used throughout except for IAT
IAT = Indirect antiglobulin test using untreated cells and anti-human IgG
Ig sc = Immunoglobulin subclass
Ig lc = Immunoglobulin light chain type

For other symbols see legend of Table 3.

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distinguish the different D phenotypes and, when correctly standardised, will lessen the dependence on the small quantities of the special polyclonal reagents available.

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We are grateful to the many colleagues who have sent us blood samples with unusual D phenotypes. We also wish to thank Dr D Crawford and Dr D Voak for supplies of UCH D4 and HD7 respectively.

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antibodies than is the D of c(D)(e)/cde Rh:33. However, of the monoclonal antibodies tested here only one, HD7, was able to detect DVI consistently. Of the IgG anti-D antibodies (Table 2) only one, UCH D4, reacts with some category VI samples. Most IgG antibodies detect category IV but REG A, and 8D6 usually, miss category IVa and IVb, and GAD 2 reacts very weakly. UCH D4 is able to make the distinction between IVa and IVb samples. Category Va cells give the same sort of variable pattern as that found with the IgM anti-D. Four antibodies, REG A, 8D6, GAD and GAD 2 miss category Va completely.

D^u cells were very difficult to detect with both the IgG and the IgM antibodies. Several D^u samples which also lack G antigen were almost impossible to detect and some samples classified as D^u by polyclonal reagents did not react with any of the monoclonal anti-D by the methods used. It is known that the strength of the D antigen of cells classified as D^u is very variable. Some D^u samples react with most anti-D by the anti-globulin test or when enzyme treated cells are used, whereas others react with only a few anti-D. Because the MRC Blood Group Unit is sent 'problem' blood samples it is likely that most of the samples tested will be on the lower end of the D^u scale, that is, those with very weak D antigens. This may explain the difficulty experienced when trying to detect these antigens.

In contrast to polyclonal anti-D, the IgG monoclonal anti-D did not agglutinate saline suspensions of homozygous -D- and .D. cells. Nor did they agglutinate D+ cells of samples with reduced sialic acid levels such as En(a-) cells.

Blocking of the D antigen was attempted by incubating CDe/cde cells with the IgG anti-D antibodies. The 'blocked' cells were then tested with the monoclonal IgM anti-D and one polyclonal anti-D agglutinin. One antibody, FOG B, was able to block the action of all the IgM anti-D but the other 10 IgG monoclonal anti-D had little or no blocking effect. This may suggest that FOG B reacts with a common epitope or, perhaps, binding to it's epitope interfers with the ability of the other anti-D to bind to a different epitope. Blocking tests with DIII, DIV, DV and DVII were attempted: the pattern of reactions was not easy to interpret. So far use of monoclonal anti-D has not revealed the number of epitopes which make up the complete D antigen.

Tests, not only with these 21 antibodies but also with many others, have revealed only one monoclonal anti-D that behaves in the same way as an anti-D made by a category D person. HD7 gives the same pattern of reactions as an anti-D made by some category DIVa individuals.

Although the work reported here involved mainly the use of agglutination tests with untreated and papain treated cells, the antibodies were used by a number of other methods. No single method was able to detect the D antigen of all known D phenotypes. However, these monoclonal anti-D can be used to

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the sub-divisions, IVa and Va, express the low frequency marker antigens Go^a and D^w respectively. Anti-Tar identifies another low frequency antigen expressed by cells with a category VII D antigen.

Category VI cells have the least D as determined by anti-D made by D- and D+ people. A different, characteristic form of weak D is associated with the infrequent Rh antigen Rh33. (For review of Rh see Issitt 1985).

The most difficult D phenotype to define is D^u. This is a term for a quantitative and not qualitative variation, a weakening of D expression. It's definition is dependent on the reagents and techniques used in a particular laboratory. Tippett and Sanger made no attempt to fit D^u samples into categories.

The monoclonal antibodies were mainly studied by agglutination tests, read microscopically. Saline suspensions (3%) of untreated and papain treated cells were used against the IgM antibodies and papain treated cells only against the IgG antibodies. Titrations (Table 2,3) of the antibodies when tested against CDe/cde cells showed the activity of all antibodies to be greatest with enzyme treated cells; the indirect anti-globulin test (IAT) did not enhance the reaction. Even for the IgG anti-D IAT gave lower titres than when papain treated cells were tested (Table 2).

Category IIIa and IIIc samples were strongly agglutinated by all antibodies. Unfortunately no category II sample was available for testing. Category VII cells when enzyme treated could also be detected by all reagents. However, HAM B did not agglutinate untreated DVII cells and 3 other antibodies reacted weakly (Table 3).

Most of the IgM antibodies agglutinated IVa and IVb cells with the exception of HAM 1 and HD7. These two reagents were not able to detect the DIV antigen even when papain treated cells were used (Table 3). Category Va was surprisingly difficult to detect especially on untreated cells. Three reagents, FOM 1, HAM A and HAM B did not detect Va; the others did so to varying degrees. The strength of reactions of some antibodies varied, from positive or weakly positive to negative, for cells from members of the same category or subdivision of a category. The order of strength of reactions was the same as that found with polyclonal anti-D.

Three antibodies, FOM A, HAM A and HAM 2, reacted with the characteristic weak D antigen of c(D)(e)/cde the complex associated with the infrequent antigen Rh33. FOM A could detect the antigen on untreated cells, the other two antibodies required papain treated cells. MAD reacted very weakly with papain treated Rh:33 cells.

DVI is usually easier to detect with polyclonal anti-D

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Monoclonal anti-D: Reactivity with various D phenotypes.

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At the MRC Blood Group Unit we have, over the years, tested blood samples from numerous individuals having unusual expression of the Rh antigen D. Therefore, when monoclonal anti-D antibodies became available, we were very interested to see how these reagents would react when tested against the different forms of the D antigen. Reported here are some of the results of testing 21 human monoclonal anti-D against cells of various D phenotypes, including D^u, Rh:33 and D categories.

19 of the antibodies discussed here were made by Drs. Hughes-Jones and Thompson and are listed in Table 2 and 3. The antibodies were produced by EBV transforming B cells from the peripheral blood of recently boosted donors and then fusing them with mouse myeloma cells to form heterohybridomas (Thompson et al 1986). The various cell lines originated from 5 donors. Where more than one monoclonal antibody has been obtained from a single donor, there is evidence that they are different (class, subclass, light chain type, migration in PAGE). The exceptions to this are FOG 3, FOG A and FOG C, which are probably the same antibody.

Two other monoclonal antibodies, UCH D4 (Crawford et al 1983) and HD7 (Lowe et al 1986), were tested in parallel. UCH D4 is an anti-D produced by an EBV transformed human lymphoblastoid cell line; the first stable EBV transformed anti-D producing cell line to be reported. HD7, a human-human monoclonal anti-D, was obtained by fusing a human lymphoblastoid line (W1-L2-729-HF2) to lymphocytes from an immunised donor.

Before giving details of the investigation a reminder of the complexities of the D antigen seems appropriate. The expression, on red cells, of the Rh antigen D can vary both quantitatively and qualitatively. People whose cells lack part of the D antigen may when immunised, make an alloanti-D. Tippett and Sanger (1962,1977) analysed results of cross-testing cells and sera of D positive individuals who had made anti-D and classified these D antigens into 6 categories. A seventh category was added more recently (Lomas et al 1986), whereas category I has been discarded. The interaction between cells and serum of people with D on their cells and anti-D in their serum is shown in Table 1.
Categories III, IV and V are now sub-divided. Cells of two of

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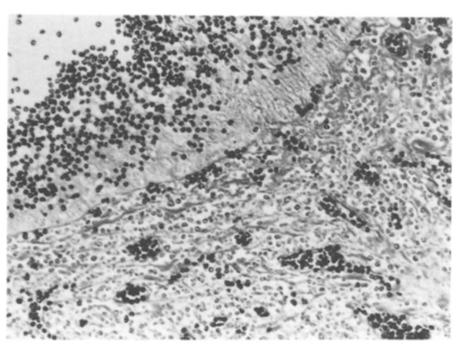

Fig. 1a

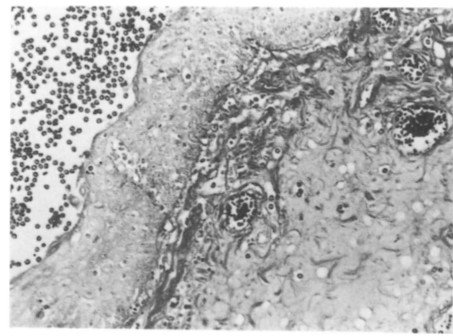

Fig. 1b

Fig. 1 Monoclonal anti-A (CMA) was used for examination of the localisation of A-group substance in esophagus mucous membranes by using MCAR method; magnification, 1x40. 1a: positive reaction. 1b: negative reaction.

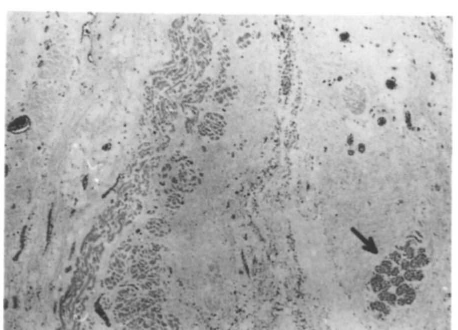

Fig. 2a

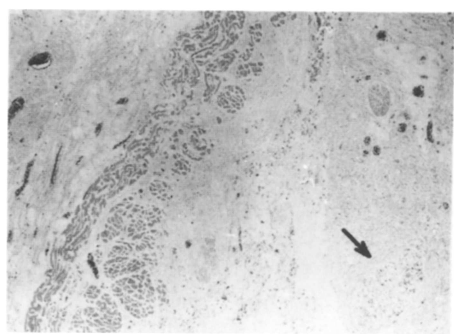

Fig. 2b

Fig. 2. Localisation of B-blood group substance in serous gland by using MCAR method; magnification, 1x40. 2a: MCAR in serous gland was positive reaction on the use of monoclonal anti-B (CMB. titer: 1:64). 2b: negative reaction on the use of anti-B serum (1:64).

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Cynomolgus) and dog (Beagle) erythrocytes whereas the CMA and SMA agglutinated only human and pig erythrocytes. Similarly commercialized human anti-B serum agglutinated erythrocytes of human B, A_1B, A_2B and pig, rabbit, dog, guinea-pig, mouse and monkey erythrocytes. CMA and SMA agglutinated only human and pig erythrocytes, and CMB and SMB agglutinated human and rabbit erythrocytes, showing that the monoclonal anti-A and anti-B antibodies have extremely high specificity, compared with the commercialized polyclonal antibodies (Table 1).

The carbohydrate chain of pig erythrocytes and human blood group A antigen is quite similar. That of rabbit and human blood group B is also very similar.

We examined the agglutinin activity of CMA by using different phenotypes, A_1 and A_2 human erythrocytes. It was obserbed that group A_1 was higher than group A_2 in the agglutination reaction. Group A_1 was also higher than group A_1B. A similar result was obtained when commercialized anti-A serum and SMA were used. These results suggest that high agglutinin titer or low agglutinin titer is related to the number of the receptor of blood group substance which is localized on the surface of erythrocytes.

The titer of CMA was lost after absorption with human secretory group A saliva, and the same result was observed for CMB and human secretory group B saliva. The activity of CMA and SMA was lost after absorption with N-acetyl-D-galactosamine, and that of CMB and SMB were also lost with D-galactose.

CMA, CMB, SMA and SMB produced a single precipitation band with anti-mouse-IgM by the micro-Ouchterlony method. The results in this study also demonstrated that the monoclonal antibodies are IgM molecules.

On the examination of bloodstain, CMA and SMA were strongly reacted with human bloodstain of A and AB, but SMA was weakly reacted with pig bloodstain. These results suggest that if we use monoclonal antibodies for examination of bloodstain, we must check the specificity of monoclonal antibodies and the origin (human or animals) of bloodstain for successful wark of forensic medicine.

In this study, CMA, CMB, SMA and SMB were used for examination of the localization of blood group antigens in esophagus mucous membranes by using MCAR method. It was clearly confirmed that the blood group antigens are localized in squamous cells of esophagus mucous membranes (Fig. 1a and 2a). The distribution of blood group substance in serous gland was examined by using CMA, CMB, SMA SMB and antisera. When the titer of monoclonal antibodies and antisera was more than 1:128, MCAR in serous gland was positive. MCAR in serous gland was negative on the use of antisera (the titer 1:64), but that was positive on the use of monoclonal antibodies (the titer; 1:64)(Fig. 2a and 2b).

The difference of specificities of monoclonal antibodies and antisera can be useful for the study on localization of blood group antigens in various tissues and monoclonal antibodies will be more useful in forensic serology in the future.

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Table 1. Comparison of agglutination reaction of antiserum (polyclonal antibody) and monoclonal antibody against erythrocytes of human and various animals

Erythrocyte	Number of Examples	Anti-A Serum[1)]	Monoclonal Anti-A Antibody[2)]	Anti-B Serum[1)]	Monoclonal Anti-B Antibody[2)]
Human A_1	100	+++	+++	-	-
A_2	8	+	+	-	-
B	100	-	-	+++	+++
O	100	-	-	-	-
A_1B	100	+++	+++	+++	+++
A_2B	6	+	+	+++	+++
Monkey:					
Rhesus	5	+	-	+	-
Cynomolgus	5	+	-	+	-
Dog:					
Beagle	5	++	-	+++	-
Horse	5	++	-	-	-
Pig	10	+++	+～++	+++	-
Sheep	5	++	-	-	-
Cat	5	++	-	++	-
Rabbit	20	+++	-	+++	+++
Guinea-pig	10	-	-	++	-
Mouse	20	-	-	++	-
Hen	3	-	-	-	-

1: Ortho Diagnostics Inc.

2: Seraclone®, Biotest-Serum-Institut GmbH and prepared by us

Agglutination grade: +++, Strongly positive; ++, Moderately positive; +, Weakly positive; -, Negative

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(Rhesus and Cynomolgus), dog (Beagle), horse, pig, sheep, cat, rabbit, guinea-pig, mouse and hen was tested with these monoclonal antibodies.

3. APPLICATION TO BLOODSTAIN

Whole blood of human and animals mentioned above was droped on the filter paper and dried up at room temperature. Bloodstains were tested by absorption-elution method.

4. ABSORPTION TEST BY HUMAN SALIVA

CMA, CMB, SMA and SMB (1:8) were absorbed with Se and se saliva which were boiled for 30 minutes before use. After the mixture had been left at room temprature for 2 hrs. and at 4°C overnight, 2 % erythrocyte suspension was added to the mixture, and the activity of the monoclonal antibodies was examined.

5. AGGLUTINATION INHIBITION TEST BY MONOSACCHARIDE

10 mM N-acetyl-D-galactosamine was added to CMA and SMA (1:8), but 10 mM D-galactose was added to CMB and SMB for absorption. After the mixture had been left at room temperature for 2 hours and then at 4°C overnight, the activity of monoclonal antibodies was measured by using 2 % erythrocyte suspension.

6. 2-MERCAPTO-ETHANOL TREATMENT

CMA, CMB, SMA and SMB were treated with 2-mercapto-ethanol in the same method as described by Grubb (1958).

7. ANALYSIS OF THE MONOCLONAL ANTIBODY BY GEL-DIFFUSION METHOD

The immunogloblin type of CMA, CMB, SMA and SMB obtained in our study was determined by micro-Ouchterlony method (1958).

8. EXAMINATION OF THE LOCALIZATION OF BLOOD GROUP SUBSTANCE IN THE TISSUES

The localization of blood group antigen in human tissues was investigated by mixed-cell-agglutination reaction (MCAR) method using all monoclonal antibodies and antisera.

RESULTS AND DISCUSSION

The specificity of the CMA, CMB, SMA and SMB which are biologically homogenous was investigated. Commercialized anti-A serum (polyclonal antibody) agglutinated erythrocytes of human A_1, A_2, A_1B, A_2B as well as pig, sheep, rabbit, cat, monkey (Rhesus and

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Specificity of the Monoclonal Antibody and its Application to Forensic Medicine

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INTRODUCTION

Studies on the molecular level of antibody are one of the most advanced in the fields of medicine and biology. Since antibody has a high-specific binding activity, it has been used in various fields as a qualitative and quantitative reagent. However, antiserum is a mixture of antibodies which react to different antigen determinant groups. This indicates that antiserum can be chemically impure. Many immunologists have attempted to obtain the antibody which recognizes a specific antigen determinant group. Köhler and Milstein (1975) succeeded in obtaining monoclonal antibody with cell fusion between myeloma cells and spleen cells of immunized mice.

We examined the serological specificity for blood group substances by using monoclonal antibodies (anti-A: SMA, anti-B: SMB) which were prepared by us, commercialized monoclonal antibodies (anti-A: CMA, anti-B: CMB) and commercialized polyclonal antibodies (antiserum).

MATERIALS AND METHODS

1. IMMUNIZATION AND CELL FUSION

We immunized BALB/c mice with human erythrocytes (5×10^8 erythrocytes per injection every 5 day over a period of 4 weeks), and checked the titer of antibody of the mice every week. In the 5th week, all the mice were sacrificed and the spleen cells were obtained. Spleen cells and myeloma cells were fused by the method of Schreier (1980) with some modifications. The procedures for the preparation of monoclonal antibodies were previously described (Ikeda and Nagai 1984).

2. SPECIFICITY OF THE MONOCLONAL ANTIBODIES AND POLYCLONAL ANTIBODIES

We examined the specificity among CMA, CMB, SMA, SMB and antisera using human erythrocytes (A_1, A_2, B, O, A_1B and A_2B) by agglutination reaction. Moreover, 2 % erythrocyte suspension of monkey

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(4) Ease of staff training (ELISA is far less complex than elution or inhibition techniques)

(5) Objectivity of results

The conjugates are a vital part of the procedure and we feel that there could be some improvement with these particular reagents.

Evaluation of each MCA requires a good deal of work and the following testing protocol is suggested:-

1. Control blood and body fluid samples
2. Control stains of blood and other body fluids
3. Ageing experiments
4. Case-work evaluation
5. Determination of negative values

Nearly all of the forensic science laboratories have to use commercially obtained reagents and this poses some of the most difficult problems.

1. How reproducible are the results from batch to batch?
2. Can we obtain sufficient quantities?
3. Can we obtain the same reagents over long periods of time?
4. Once characterised can we rely on the company to keep the reagent consistent?
5. If there are additives, will the company tell us what they are?

When an individual laboratory obtains a MCA which they consider to be suitable for work in forensic science it would be of considerable value to provide this information to their colleagues, at home and abroad, as soon as possible. Also, if all of the forensic science laboratories will be using the same reagents it will be necessary to decide a testing protocol to which we could all adhere.

However there still remains the problem of how to disseminate the information and we suggest a sub-group of the haemogenetics society with a representative in each of the member countries who would be responsible for informing his colleagues.

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It is of interest to note that some of the MCAs will detect the ABH antigens in bloodstains far better than those in body fluid stains and the converse is also true. This is not surprising as the oligosaccharide structures are different one from the other and it may prove to be of considerable advantage when typing stains which are mixtures of blood and other body fluids.

The Lewis MCAs which we have been testing have shown some rather surprising results in that they appear to be detecting a straightforward difference between secretors and non-secretors rather than Le(a) and Le(b) types. Again this can be explained by the differences in the basic oligosaccharide structures and may be of considerable benefit in forensic science because we are able to distinguish between the Le(a-b-) secretors and non-secretors. However these particular MCAs do not provide very good results with the typing of whole cells and using other MACs which detect Le(a) and Le(b) could cause problems with reporting for court purposes.

Although there have been many publications on the use of a MCA for the typing of Gm(3) from dried blood we have not attempted to assess its value. This is for 2 reasons. Firstly this is not a good marker for discrimination purposes within a white caucasian population. Secondly, we use the presence of Gm(3) to confirm the absence of Gm(1) and Gm(2) such that we can report a Gm(-1,-2) phenotype. If we were to use a very sensitive method for the detection of Gm(3) by the use of a specific MCA, and still detect Gm(1) and Gm(2) by polyclonal antisera we would defeat the object of the exercise.

Discussion

It is becoming clear that there is a considerable advantage in using MCAs linked to an ELISA detection system:-

(1) Specificity of each MCA
(2) Large volumes of reagent
(3) Longer 'shelf-life' of the reagent

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In general this method has worked well with no cross-reactivity being observed although a few tests gave inconclusive results. The method is not as quantitatively reproducible as that using MHS-5 and this could be as a result of the more complicated testing routine.

Neither anti-p30 nor MHS-5 were found to cross-react with vaginal secretion or any other body fluids but the system was not able to be used to determine the post-coital interval. This could be due to many factors.

Case-work trials are now underway at the MPFSL and it is expected to use them for routine testing in the near future. They will be the subject of future publications.

Now that we have reliable systems for detecting semen, it would be desirable to obtain MCAs which are specific for vaginal secretion, urine, faeces and sweat.

Grouping of blood and body fluids

A number of surveys have been carried out whereby MCAs have been compared with polyclonal antisera using the traditional inhibition and elution systems for blood and body fluid grouping.

In most of our initial testing we encountered problems with the red cells adhering to the plastic tubes and thus rendering the inhibition testing unworkable.

With the elution system the MCAs which were available to us provided problems with very strong antigen-antibody binding such that heat elution was prevented.

Subsequent testing using an ELISA method has shown an enormous increase in the reliability and sensitivity of the detection of the ABH antigens. There is still a problem with AB stains but it is felt that this is not insurmountable.

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In the UK, Fletcher and co-workers at the Central Research Establishment have pioneered the use of MCAs and ELISA in forensic science, but we are also aware that this work is also being pursued in many other countries.

At the Metropolitan Police Laboratory we have had approximately 18 months experience with the use of MCAs and ELISA for the detection of semen and also for use in the ABH, Lewis and MN blood group systems.

Identification of Semen

Two MCAs have been used in this context, both of which were obtained from the U.S.A. These detect proteins which are present in seminal plasma and are therefore unaffected by the presence or absence of spermatozoa.

Seminal Vescicle Antigen

The MCA which recognises this antigen is termed MHS-5 (Mouse anti-Human Semen-5) and is used for the detection of epitopes on small peptide fragments in the seminal plasma. Initially it was used in conjunction with a polyclonal mouse IgG-horse radish peroxidase (HRP) but cross reactions were observed with samples of saliva from both males and females.

Biotinylated MHS-5 with steptavidin-HRP as conjugate eliminated this cross-reactivity and specificity for semen of man and higher primates, was achieved.

p30

This is a protein produced in the prostate and has been used for some considerable time as a seminal plasma marker by immunoprecipitation with a polyclonal antibody produced in rabbits.

The testing system which we have used employs initially MCA to p30 followed by a polyclonal anti p30-alkaline phosphatase conjugate.

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The Use of Monoclonal Antibodies in Forensic Science

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Introduction

Although the advent of DNA profiling in forensic science is an enormous step forward for the identification of those involved in crime, it will still be necessary to identify the types of stains and on many occasions the red cell antigens and enzyme polymorphisms will have to be detected.

This is especially so when grouping semen stains in which there are few or no spermatozoa. In this situation it is first necessary to identify the presence of semen using biochemical markers which are present in the seminal plasma, followed by ABH grouping and the determination of other genetic markers which are present in the stain extract and also in the control samples obtained from suspects and victims.

The time taken for the analysis may also be an important factor when the police are in a hurry to eliminate suspects from their enquiries. In such cases, if a person cannot be eliminated by traditional grouping, there could be justification for the continued detention of the suspect pending DNA analysis.

The introduction of monoclonal antibodies (MCA) for detection purposes and grouping procedures was inevitable. MCAs have many theoretical advantages over their polyclonal counterparts, not least of which is their monospecificity. Other advantages include the supply of large volumes of reagent which should be available over long periods of time (theoretically forever) and the ability to pick specifities which have been hitherto unobtainable.

Many problems have been experienced with the use of MCAs in the absorption-inhibition and absorption-elution methods which have been used for many years in forensic science laboratories, and it has become apparent that the ELISA technique could be a considerable improvement.

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reactions were due to crossreaction with "N" were excluded by using S positive red cells that were chymotrypsin treated prior to the trypsin treatment.

General Comments on MN Reagents

Correctly standardised monoclonal anti-M and anti-N reagents are excellent red cell typing reagents. Furthermore, we suggest that some monoclonal anti-M reagents eg BS 57 and BS 38 (with neuraminidase treated material) are sufficiently specific to be developed for forensic use, perhaps by ELISA techniques.

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Table 13. Monoclonal Anti-M BS 44 - Specific by Dilution

	Spin-Tube Titres RT PH 8.0		
	Saline	5% Albumin	Trypsin
3% RBC			
MM	128,000	256,000	2,000
MN	64,000	256,000	2,000
M^VN	128,000	256,000	N/T
M^CN	2,000	4,000	N/T
NN (3)	1,000-4,000	4,000	128-1,024

Dilution to 8,000 - Negative with NN - Is also negative with M^c red cells.

The data in Table 14 shows a third type of anti-M (BS 38) that only behaves as a specific anti-M with neuraminidase treated red cells, while it gave greater reaction with N than M in other types of tests. This suggested that the N reaction was sialic acid dependent (Bird and Wingham 1970) on the α chain as it was not destroyed by chymotrypsin.

Table 14. A Monoclonal Anti-N>M (BS 38) - Which is an Anti-M With Neuraminidase Treated Red Cells

	Spin-tube titres RT at pH 5.0			
	Saline	Neuraminidase	i Chymotrypsin ii Trypsin	Chymotrypsin
3% RBC				
MM	4,000	512 - 1,000	128 - 4000	16,000
MN	8,000	128	8,000	16,000
M^V	N/T	128	N/T	N/T
M^cN	N/T	128	N/T	N/T
NN	16,000	0	16,000	32,000

Trypsin Sensitivity of MN Antigens (α chain)

The results of our studies confirmed the trypsin sensitivity of M reactions using the monoclonal anti-Ms, BS 57 and BS 44. However, with BS 38 the crossreactive anti-N>M antibody demonstrated trypsin resistant MN epitope(s) on the α chain. The possibility that these

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Thus, the δ "N" of MMss (S negative) red cells appears resistant to the action of chymotrypsin whereas it is reduced or destroyed in S positive red cells. It is possible that the development of S on δ causes unfolding of the sialoglycoprotein which reveals sites sensitive to chymotrypsin.

Anti-M Reagents

The M antigen on red cells is all the end product of the M gene. Therefore it is possible to have specific anti-M reagents that do not react at all with N. A good example of a specific anti-M antibody is BS57 (Table 12) which did not agglutinate and was not absorbed by NN red cells. Its failure to react with the M^c variant suggests glycine is an important part of the M antigen (Dahr 1977) recognised by this monoclonal anti-M. A specific anti-M monoclonal antibody that also detects M^c does exist (J. Moulds, personal communication).

Table 12. A Specific Monoclonal Anti-M (BS 57)

	Spin-Tube Titres		RT at pH 8.0
	Saline	Trypsin Destroyed	Chymotrypsin Enhanced
3% RBC			
MM	16,000	0	64,000
MN	16,000	0	64,000
M^vN	16,000	0	64,000
M^cN	0	0	0
Control			
NNSs	0	0	0

Amino acid sequence

Antigen	Position 1		Position 5
M	Serine	M^c (Serine — Glutamic acid)	Glycine
N	Leucine		Glutamic acid

Other monoclonal anti-M antibodies that show some crossreaction with N but greater reactions with M, can, like anti-N reagents, be made specific by dilution, as shown by the example with BS 44 in Table 13. This "specific" anti-M reagent fails to detect M^c red cells which gave only the same reactivity as NN red cells. The reactions of BS 44 are enhanced with M-positive red cells suspended in 5% albumin. However, the reaction with NN red cells is not enhanced, perhaps because the crossreacting antigen site density is much lower than that of the M sites.

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Table 10. Monoclonal Anti-N Reagents - "Specific" by Dilution

	Spin-Tube Titres		RT at pH 8.5	
		Monoclonal Anti-N		
	BS 75 Ac.	BS 41 Ac.	NM 00286 Sup.	N3 Sup.
Source	Sonneborn		Moulds	Fletcher
Dilution "Specific"	4,000	500	32-64	3
3% RBC				
NN	128,000	4,000	256	16
MN	32,000	4,000	128	8
Controls*				
MMSS	1,024	256	16	1
MMSs	1,024	256	16	2
MMss	256	128	16	<1

*S+ show greater cross reaction - 30% more δ "N"

Three of the anti-N antibodies gave greater "N" reactions with S positive than S negative MM red cells. Therefore it is important to use S positive MM red cells as the negative control test for standardisation of the "specific" anti-N reagent dilution and also for N typing.

Chymotrypsin Effect on δ "N" is Reduced in ss Genotypes

The effect of chymotrypsin for destroying δ "N" reactions gave surprising results (Table 11) as some reactions were enhanced, presumably because enzyme enhanced agglutination occurs if the antigens involved are not destroyed by the enzyme.

Table 11. Ss Type Affects δ "N" Reaction With Chymotrypsin

	Titration Scores		Spin-Tube		RT at pH 8.5	
			Monoclonal Anti-N			
	BS 75		BS 41		NM 100286	
	Sal.	Chymo-trypsin	Sal.	Chymo-trypsin	Sal.	Chymo-trypsin
3% RBC						
MMss	69	74	40	31	22	40
MMSs*	83	33	48	0	23	16

*SS gave similar results

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Table 9. MNSs Glycoproteins - structure and site density

Chain	α (Glycophorin A)		δ (Glycophorin B)
Antigens	M N		"N" S s
	Genotype		
No. of sites on each chain	MM, NN 1,000,000		"N" 250,000 30% more if S+
	MN 500,000		
N + "N"	NN 1,250,000	if S+	1,325,000
α	MN 750,000		825,000

The M and N genes control the production of M and N on the α sialoglycoprotein, but an "N" (N-like) structure is also produced in the absence of an N gene on the δ sialoglycoprotein. This "N" structure is responsible for the "crossreaction" of anti-N reagents with the δ "N" on the red cells of people of the homozygous genotype MM. Furthermore MM people who are also S positive have more "N" than MMss types as S positive people have about 30% more δ (Dahr et al 1978).

The effect of trypsin and chymotrypsin is also useful in the characterisation of anti-M and anti-N reagents as they cleave the α and δ chains respectively (Dahr et al 1975).

Anti-N Reagents

Because anti-N antibodies detect δ "N" it is only possible to make anti-N reagents "specific" by dilution, as shown in Table 10. These reagents are only specific at selected dilutions for detecting the product of the N gene by appropriate agglutination tests. However, these reagents are not specific in absorption and elution tests as the antibodies still bind to "N" antigens. The reagent's specificity in agglutination tests is a function of the amount of antibody and the relative antigen site density. Thus, at a suitable dilution there is insufficient IgG anti-N to agglutinate red cells of relatively low site density such as MM with only δ "N" sites.

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Table 8. Tests for Weak D(D^u) With an IgM (MAD-2) + IgG Anti-D Blend*

	1 : 1 vols - Spin-Tube Tests X 3% RBC						
	Weak D(D^u) Samples						
	1	2	3	4	5≠	Controls	
Method	$R_1^u r$	$R_2^u r$	$R_2^u r$	$R_2^u r$	$R_1^u r$	R_1^r	rr
Immediate Spin							
Saline	wk	+	wk	+++	(+)	++++	0
15 min 37°C							
Saline	0	+	0	+++	wk	++++	0
AHG	+++	+++	+++	N/T	+++	N/T	0

* Saline titre 1,024 x R_1r (Pool of 4)

≠ Previously grouped as R_1r with an albumin anti-D.

Thus this new approach to anti-D reagents using a high titre IgM monoclonal anti-D represents a considerable improvement over conventional IgG anti-D reagents as it simplifies routine Rh D typing work. The expected incidence of discrepant types in the saline test phase of about 0.5% is regarded as acceptable and can be dealt with by using the antiglobulin phase in the same tube.

MN MONOCLONAL ANTIBODY REAGENTS

The production of mouse monoclonal anti-MN reagents by many workers (Frazer et al 1982, Sonneborn et al 1984, 1987, Wasniowska et al 1985, Nichols et al 1985, Fletcher et al 1986, Rubocki and Milgrom 1986) has overcome the problems of short supplies of MN reagents. However, red cell serologists must not assume that monoclonal anti-M/N reagents will behave as reliably as conventional reagents in physiological saline. Experience has shown that each monoclonal anti-M or anti-N must be carefully standardised to identify its optimum pH as well as the degree of crossreaction at various temperatures with the various MN phenotypes.

Monoclonal anti-M/N antibodies are mainly IgG types but some are IgM (Wasniowska et al 1985). The ability of IgG anti-M/N to cause agglutination in saline tests is due to the high number of M, N, "N", sites on red cells, as shown for the various phenotypes in Table 9 (calculated from data reviewed by Anstee 1983).

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laboratory in comparison to an albumin anti-D reagent. The activity of IgM anti-Ds can be enhanced by using 2-stage enzyme treated cells (Table 6) but this would mean setting up a second test and is time consuming.

A New Approach to Anti-D Reagent Production

MAD-2 IgM anti-D was shown to be able to agglutinate R_1r red cells, even though they were previously sensitised with high levels of IgG anti-D (Table 7). Thus MAD-2 with a saline titre of 1,024 is far more resistant to the blocking action of IgG anti-D than a polyclonal IgM anti-D that had a typical titre of only 16.

Table 7. Blocking of High Titre IgM Anti-D (MAD-2)
I. By Prior Sensitisation With IgG Anti-D

	2 Volumes IgG Anti-D : 1 Volume 3% R_1r RBC				
	Level (IU/ml) of IgG Anti-D (GD110)				
	125	62	31	15	Saline Control
IgG Anti-D					
Sensitised R_1r	0	0	0	0	0
2nd Stage					
IgM Anti-D added*					
Polyclonal (2300)	0	Weak	$+^w$	$+^w$	++
Monoclonal (MAD-2)	0	+	++	+++	++++

*1 Volume IgM anti-D added to sensitised R_1r cell button after removal of IgG anti-D supernatant.

Therefore a new anti-D reagent approach was possible by blending the monoclonal MAD-2 IgM anti-D with a small amount of selected polyclonal IgG anti-D. This reagent blend was designed to provide a single anti-D reagent that could detect normal RhD positives by simple saline tests and only use an enhancement test, antiglobulin (AHG) test for the detection of weak D types in donor blood typing as we considered it unnecessary for patient Rh typing except in the case of discrepant results (Moore 1984, Voak 1986).

The final MAD-2 blend with IgG anti-D (IgG fraction) strongly agglutinated all normal Rh D phenotypes and most variants (except Category VI) by saline tests, even using rapid immediate spin techniques. The reagent had a saline titre (5 minute spin-tube) of 1,024 x R_1 (pool of 4) red cells and the results of saline and AHG tests with weak D (D^u) samples are shown in Table 8. The amount of IgG anti-D fraction added was just sufficient to give reliable AHG test detection of Weak D (D^u) red cells and did not reduce the saline test agglutination of the neat reagent with R_1r cells (weakest common Rh D positive phenotype). A minor blocking of weakly saline agglutinated D^u types occurred on incubation after 15 minutes at 37°C, but prolonged incubation does not reduce the saline agglutination with R_1r red cells.

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Virus (EBV) transformed human B lymphocytes. Monoclonal IgG anti-D antibodies have not yet offered advantages over polyclonal anti-D reagents, as the yields are not economic and they miss weak D(D^u) and some D variants (Voak 1986).

However, progress has been made on stabilising anti-D secreting cell lines by fusing them with a human-mouse heteromyeloma (Bron et al 1984) or a mouse myeloma (Thompson et al 1986), and Thompson et al have produced an excellent IgM anti-D that is suitable for reagent use. The tissue culture supernatant of this IgM anti-D (MAD-2) has a saline test spin-tube titre of at least 1,000 with red cells of all the common RhD positive phenotypes and it is sufficiently potent for use by slide or immediate spin-tube tests.

Reactions of Monoclonal Anti-Ds with Weak D and D Variants

The data in Table 6 shows the behaviour of various monoclonal anti-D antibodies with D variants and weak Ds. The IgM anti-D of Lowe et al, 1986, is not as potent as MAD-2, but is scientifically interesting as it misses a Category IV variant detected by MAD-2.

Table 6. Spin-Tube Test Reactions of IgM Anti-D Monoclonals with D Variants and Weak D (D^u) Red Cells

Conventional Anti-D								
% Positive		100	96	74	35	97		
Category		III	IV	V	VI	VII	$R_1{}^u r$	$R_2{}^u r$
Number tested		2	1	1	3	1	9	7
HD7 (IgM)	Saline	V	0	V	0	+	0/W	0/++
	Papain	C	0	C	++/C	+	+/V	++/C
MAD-2 (IgM)	Saline	V	C	+	0	0	0/++	0/V
	Papain	C	C	C	0	+	++/V	++/V
D4 (IgG1)	Papain	+++	+++	+++	0	+	0/++	0/++
GAD (IgG3)	Papain	+++	0/W	0/W	0	+	N/T	N/T

? Category IV is D^{abCD}, so HD7 detects D^A and/or D^B.

Monoclonal anti-Ds showing different patterns of reaction with the various categories of D variants (Tippett and Sanger 1962, Race and Sanger 1975) undoubtedly provide an avenue of research to help elucidate the fine structure of the D antigen complex.

The failure of IgM anti-Ds to agglutinate some weak D (D^u) and D variant types is a limitation of an IgM only type of reagent. MAD-2 gave 79 (0.5%) discrepant results out of 14,680 Rh D typing tests using a microplate method in our Cambridge pre-natal testing

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Table 5. Monoclonal Reagents Agglutinating Both A and B Cells That May be Used Instead of Anti-A,B Serum Reagents

	Anti-A,B ES.15	Anti-A,B ES.15 + Anti-B 5A5	Anti-A MH04 + Anti-B 5A5	Conventional Commercial X (USA) O Serum
2% Cells				
A_1	V	V	C	C
A_2	V	V	C	C
B	(+)*	V	C	C
A_2B	V	V	C	C
A_3 (5439)	++	++	V	++
A_X	+/++	+/++	++	++
B_W	-	++	++	++
O Cells	-	-	-	-

*Weak reactions with B cells and negative with B weak cells

C	V (+++)	++	+	(+)	GW	W
One Clump	Several Clumps	Smaller Clumps	Granules	Small Granules	Microscopic Cells/Clumps (8-12)	(4-6)

Economy of Automated ABO Grouping (Technicon Autogrouper 16C)

MH04 anti-A at a 1,500 dilution of the supernatant detects all A subtypes down to "A_3B" (weak A_2B) but not A_x. A more concentrated 1:50 dilution of MH04 anti-A was used in an anti-A,B blend with a 1:50 dilution monoclonal anti-B (5B2) to detect weak subgroups of A and B. This anti-A,B and reverse grouping also confirms the ABO groups. This procedure enables us to identify A_x donors for research purposes as we did not want them to be labelled as group A donors. The monoclonal anti-B (5A5) was used at a 1:100 dilution.

Accurate results were obtained with these reagents and no false results were obtained in 113,703 tests with the anti-A, 135,039 tests with the anti-B and 47,259 tests with the anti-A,B. The economy of these selected monoclonal anti-A/B antibodies, enhanced by methyl cellulose (0.6-1.0%) and bromelin (0.25%) is at least five times better than with conventional reagents and offers complete reliability of donor blood typing.

Rh D MONOCLONAL ANTIBODY REAGENTS

The first monoclonal IgG anti-D was an IgG antibody produced by Crawford et al (1983). Most workers experienced difficulties in stabilising the anti-D secreting cell lines which were Epstein Barr

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Table 4. Saline Titres of Tissue Culture Supernatant Monoclonal Anti-B Reagents

	Anti-B Reagents				
3% Cells Saline RT	NB1/19	3B4	5A5	5B2	Group A Serum Commercial X (USA)
A_1B	256	64	256	128	64
A_2B	512	128	512	256	64
B	512	128	512	256	128
B Cord	256	64	256	128	64
B Weak*	0	32	64	32	64
A_1	0	0	0	0	0
O	0	0	0	0	0

*Similar results obtained with 25 additional sub-groups of B

The detection of weak B variants in 25 samples kindly donated by Dr. Leong and Mr. Mak, Hong Kong, (not classified, as saliva not tested) by the anti-Bs 3B4, 5A5 and 5B2 but not by NB1/19, which has a similar titre with A_1B and B cord cells, suggests that weak B variants have a different B specificity to normal B cells.

Anti-A,B Reagents for the Detection of Weak Subgroups of A and B

Several monoclonal anti-A,B antibodies have now been described (Voak et al 1983, Messetter et al 1984 and Moore et al 1984). One example (ES 15) by Moore et al (1984) reacts well with all group A types down to and including A_x, but only reacts weakly with even strong group B cells. Thus it must be blended with a potent monoclonal anti-B to make an anti-A,B reagent. Anti-A,B reagents can also be made by blending a monoclonal anti-A (MHO4) that sees A_x with a monoclonal anti-B (5A5) that sees weak B variants, as shown in Table 5.

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careful screening with the first batch of Bioclone anti-A gave a variable incidence (> Negroes) from as high as 0.9% (Beck) to 0.1% (Mohn). Reproducibility of reactions was poor and many centres did not find any B(A) cases.

6. Occurs due to production of some A by elevated B transferase levels in group B persons.

Quality Control of Anti-A Reagents to Avoid B(A) Reactions

Most reagent manufacturers will soon have superior anti-A monoclonal reagents. Therefore it is important to realise that this type of reagent development is not prevented by the B(A) phenomenon, as B(A) reactions are a function of anti-A concentration that can be quality controlled with B(A) or papainised B red cells. The MH04 anti-A diluted at 1/90 is still a superior anti-A reagent negative with the B(A) papainised B and O control cells by carefully read spin-tube tests (Table 3). The ability to detect weak A_x red cells is reduced but the 1/90 MH04 reagent still detects more examples of A_x than the excellent BRIC 131 anti-A of Dr. Anstee and the FDA licensed conventional anti-A,B control reagent.

Table 3. The B(A) Phenomenon is Prevented by Dilution of MH04 Anti-A and the Reagent Still Detects Many Weak A Variants

Spin Tests		Number of Variants Detected			
		Anti-A Reagents			Anti-A,B
Sub Group	Number Tested	Bioclone Ortho	MH04* 1/90	BRIC 131 Sup.	Conventional
A_x	24	17	14	9	7
A_3	6	6	6	6	6 (1 Weak)
"A_3B"	8	8	8	8	8
A_x B	1	1	1	1	1
Control A in B					
Papain	B	1+	0	1+ weak	++++

*Also negative with B(A) that is ++ with Bioclone.

Anti-B Reagents

Monoclonal anti-B antibodies (Sacks and Lennox 1981, Voak et al 1983, Messetter et al 1984) are now in widespread use as typing reagents and careful blending of some of the examples shown in Table 4 has produced monoclonal anti-B reagents at least equal to conventional FDA licensed reagents.

None of these monoclonal anti-Bs detected acquired B and they are useful for studying acquired B cases in forensic work.

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the MHO4 anti-A. The agglutination with these B cells was extremely fragile and best observed by very gentle agitation (or tip and roll) spin-tube techniques, as shown in Table 2, and the reactions were enhanced by enzyme treatment of the red cells. We then demonstrated that the reaction with these B cells was an A reaction as it was inhibited by the A but not B substance of secretor salivas.

Table 2. The B(A) Phenomenon - Method Sensitivity of MHO4 Anti-A Reaction with Group B(A) Red Cells

		Group B donor RBC					Reactive Group B		Group O
Test		1	2	3	4	5	23509	21865	Control
Spin	Saline	0	0	0	0	0	+	+++	0
	Papain	+	+	+	++	+++	++	+++	
Sed.	Saline	0	0	0	0	0	0	(+)/+	0
	Papain	W	0	W	0	0	W	++	0

The B(A) Phenomenon - B Transferase May Make Some A

Thus, all B cells have a little A that is not the product of an A gene. B cells reactive in saline tests were called B(A) red cells and the reaction with MHO4 anti-A was called the B(A) phenomenon. We suggest that B(A) red cells have A levels approaching that of weak A_x cells while the majority of B red cells have a much smaller trace of A. One of us (D.V.) contacted Dr. Watkins with this news and she was delighted to inform me that our work had provided the serological confirmation that B transferases could make some A "in vivo" as suggested by their earlier "in vitro" immunochemical studies (Greenwell et al 1979).

Characteristics of B(A) Reactions

(For reviews see Voak et al 1987 and Treacy and Stroup 1987)

1. Shown by high concentrations of certain monoclonal anti-As that see A_x.

2. Also shown by anti-A of certain high titre group O sera after absorption of anti-B and anti-AB crossreacting antibodies.

3. Best detected by gently read spin-tube tests as the agglutinates are extremely fragile and method sensitive.

4. The reactions are enhanced (variable) by enzyme treatment of the red cells, but still fragile and method sensitive.

5. The incidence of B(A) reactions in saline tests is very low as routine manual or automated grouping methods involve sufficient agitation to destroy the B(A) fragile agglutinates. Very

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The saline titres (Table 1) demonstrate the superior titres of the anti-A (MH04) compared to the monoclonal anti-A (3D3) that reacts about as well as a polyclonal FDA licensed serum anti-A reagent. (Lowe et al 1984)

Table 1. Saline Titres of Tissue Culture Supernatant Monoclonal Anti-A Reagents

	Anti-A Reagents		
3% Cells Saline RT	3D3 Neat	MH04 Neat	Group B serum Commercial X (USA)
A_1	1024	1024	512
A_2	512	512	256
A_1B	512	512	512
A_2B	64	256	64
A_2B Weak	4	256	8
A_3B	0	256	1
A_3	4	256	4
A_X	0	64	0
B(A) +++	0	32	0

Saline tests negative x B, O, A_m and A_{end} RBC

MH04 is the most powerful anti-A we have examined and it gave macroscopic reactions with 20 out of 24 examples of A_x. Absorption and heat (56°C) elution studies with MH04 confirmed it was an anti-A and not some form of anti-A,B antibody as it was not absorbed or eluted from B or control O cells. The strength of binding of MH04 absorbed by A_1 red cells is so strong that it is not eluted from A_1 cells, although it does elute from A_2, A_2B and A_x red cells. Thus the selection of monoclonal antibodies for blood grouping based on high avidity criteria produces reagents unsuitable for use by classical forensic elution techniques.

We suggest that forensic workers use these avid reagents by ELISA methods or select monoclonal antibodies to lower avidity criteria.

MH04 anti-A was used in thousands of automated and manual blood grouping tests using the UK sedimentation tube technique read after pipette transfer of the cell button to microscope slide, and no reactions were observed with B or O red cells. However, later in 1985 MH04 and 3D3 anti-As from Celltech were blended to produce Ortho's Bioclone Anti-A and after some months it became apparent that this reagent reacted weakly with a low incidence of "normal" group B red cells. Samples of some of these reactive group B bloods were referred to D. Voak by D. Davies (Ortho) and M. Beck (Kansas City) and we demonstrated that the reactive B red cells were reacting with

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THE APPLICATION OF MONOCLONAL ANTIBODIES FOR THE DEFINITION OF GENETIC MARKERS OF HUMAN RED CELLS

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INTRODUCTION

Monoclonal antibodies have now been produced to the red cell antigens A, A_1, B, A and B, M, N, D, LW, e, H, I, Le^a, Le^b, P, P_1, K, k, LKE, Lu^b, T, T^n, Wr^b, and MER 2.

Selected stable cloned cell lines that produce high yields of useful monoclonal antibodies offer considerable advantages to reagent manufacturers by providing unlimited quantities of high quality reagents (Kohler and Milstein 1975)

The aims of this paper are to describe:-

(1) the serological behaviour of selected monoclonal antibodies to ABO, RhD and MN red cell antigens that have proved themselves at least equal or superior to conventional reagents.

(2) new types of problems encountered during the use and or development of these reagents that require special quality control techniques or a new approach to reagent production.

ABO MONOCLONAL ANTIBODY REAGENTS

ABO grouping reagents should meet FDA standards (Hoppe 1979) for speed of reaction and titre, and they must be stable and specific. Selected mouse monoclonal anti-As, anti-Bs, and anti-A,B antibodies achieve these criteria and are in widespread use as blood grouping reagents (Voak 1986). These IgM anti-A/anti-B antibodies are potent red cell haemolysins in the presence of the complement of fresh serum. Therefore, the reagents must be formulated with EDTA (0.01m) at pH 7.1-7.3 for use as typing reagents.

Anti-A Reagents

By 1984 superior monoclonal anti-As had been produced that exceeded conventional anti-A reagent specifications by reacting with A_x in addition to being superior with weak A_2B "A_3B" bloods (Voak 1986, Voak and Lennox 1986, Messeter et al 1984) and based on the recent (Paris) ISBT monoclonal antibody trials and personal contacts we now know of 10 monoclonal anti-As that see A_x.

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[3]Gamma Biologicals, Houston
[4]Red Cross Blood Transfusion Service, Sydney

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SOME RECENT ASPECT ON HYBRIDOMA TECHNOLOGY

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Hybridoma technology, i.e. the fusion of an antigen-specific B-lymphocyte with an appropriate myeloma cell line, has resulted in a vast number of different reagents (monoclonal antibodies, MAb) with specificity for such molecules as enzymes and hormones, external and internal structures of bacteria, viruses and eucaryotic cells. So far, the majority of these reagents have been of mouse or rat origin, due largely to easy access to laboratory animals for immunization and the availability of myeloma cell lines. For *in vitro* diagnostic and scientific purposes these MAb have proven excellent tools with a high degree of reliability. For clinical use, however, these animal proteins harbour the danger of inducing immune responses as hypersensitivity and neutralizing antibodies. Thus, different methods are being developed to avoid these undesirable effects, including attempts to produce human MAb or make them as human as possible. Obviously, it would be most logical to fuse antigen-specific human B-lymhocytes with human or mouse myeloma cells. This approach works, but has limitations particularly in the genetic stability of the resulting hybridomas. In another approach one tries to immortalize human B-cells with Epstein-Barr-virus, but here also the stability of the clones is generally not sufficient. There remains the use of gene technology, transfecting eucaryotic cells or bacteria with genomic DNA carrying the information for the antigen binding site from mouse in combination with the constant part of the molecule of human origin. This last approach is still very much matter of experimentation and its presumed merits under debate.

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III. Monoclonal Antibodies

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CONCLUSION

The results indicate that PLG*QO is an inheritable allele. It is apparently population specific, since it has only been identified in whites. Individuals with PLG*QO have depressed PLG levels identified in fresh samples when tested by conventional radial immunodiffusion methods. Because the gene (PLG*QO) has a frequency of 0.0035 single indirect exclusion in cases of disputed parentage should be further investigated with additional genetic markers, quantitation and pedigree analysis.

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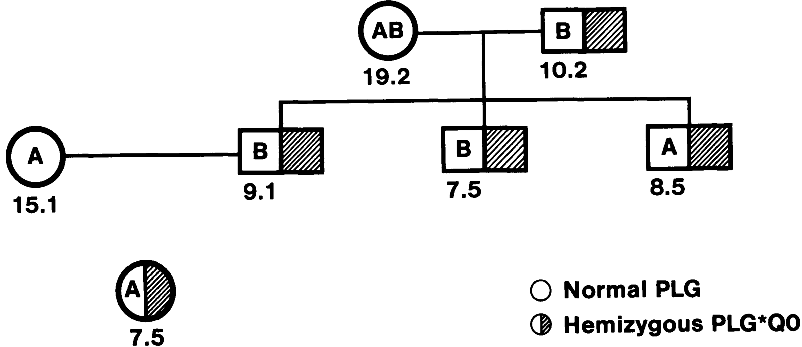
TABLE 2. MATERNAL EXCLUSIONS OBSERVED

PHENOTYPES		
CHILD	MOTHER	NO. OBSERVED
A	B	13
A	A3	1
B	A	18
	TOTAL	32

Single indirect paternal exclusions had been noted in 20 cases, most of which had high residual probabilities of paternity when the PLG exclusion was not considered. In those cases in which HLA testing was subsequently done no additional exclusions were observed and the residual probability increased. In one such case a family was obtained and appeared to demonstrate inheritance of the PLG*QO allele, figure 1. Testing of the family showed that the child in question, the alleged father and sibs and his mother all had depressed PLG levels. PLG levels of the suspected carriers ranged from 7.5- 10.2 mg/dl compared to a mean of 13.1 mg/dl in our control population of 49 donors (range 9.8 - 17.5, s.d. 1.75).

FIGURE 1.

PLG*QO Pedigree, Phenotypes and Quantitations (mg/d l)



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Calculations for the frequency of silent alleles used a formula provided by Dr. Henry Gershowitz, Department of Human Genetics, University of Michigan.

$$S = \frac{\text{frequency of parent-child incompatibility}}{\text{sum of heterozygotes}}$$

RESULTS AND DISCUSSION

Phenotype and gene frequency results are seen in table 1. The new variants PLG*M01 and A4 are further described by Dykes et al at this conference and focus slightly anodal to PLG B and A3 respectively.

TABLE 1. DISTRIBUTION OF PLG PHENOTYPES AND ALLELE FREQUENCIES IN MINNESOTA WHITES

PHENOTYPES	NO. OBSERVED	ALLELE FREQUENCIES
A	9664	A=0.6777
AA1	20	A1=0.0007
AA3	476	A3=0.0176
AA4	78	A4=0.0026
AB	7797	B=0.2860
AM01	101	M01=0.0035
AM1	13	M1=0.0004
AM5	323	M5=0.0115
A1B	11	* Q0=0.0035
A3	13	
A3B	219	
A3M01	3	
A3M5	6	
A4	2	
A4B	22	
A4M1	2	
A4M5	2	
B	1824	
BM01	36	
BM5	143	
M1	2	
M1M5	1	
M5	1	
TOTAL	20759	

* Not used in calculating other gene frequencies

The paternity cases in this study demonstrated as of December 1986 a total of 32 apparent maternal exclusions, table 2. In all cases PLG was the only incompatibility. Based upon these results and the number of heterozygotes observed in the population the frequency of PLG*Q0=0.0035.

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Incidence of the PLG*QO Allele in Human Populations.

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Knowledge about genetic variation at the plasminogen (PLG) locus has increased considerably since the independent discoveries of Hobart (1979) and Raum (1979) which described two common alleles. A recent publication by Skoda et al (1986), resulting from a 1984 symposium in Munich, West Germany, established a proposed nomenclature for the PLG system by recommending that the common alleles be called PLG A and PLG B. Eleven (11) additional variants identified by other contributing laboratories received a new name PLG*A1, A2, A3, M1, M2, M3, M4, M5, B1, B2 and B3.

Dysfunctional and fetal forms of PLG have been identified (Aoki et al, 1978; Aznar et al, 1980; Miyata, 1982; Dykes et al, 1984), but are generally associated with clinical abnormalities or, in the case of infants, transitional forms which disappear at 1-2 years of age (Dykes et al, 1984). After testing 11,071 white, 1156 black and 443 amerindian paternity cases we identified a null allele, PLG*QO, present only in whites and which generally appeared as maternal exclusions. This report documents the presence of the null allele by observations on apparent maternal and paternal exclusions, family data and quantitative testing.

MATERIALS AND METHODS

Bloods for paternity cases were drawn in ACD anticoagulant and stored at -20 until tested. Samples were tested with 14-16 genetic marker systems using a battery of RBC antigens and enzymes and serum proteins with a power of exclusion of P = 0.97 - 0.98. In selected cases HLA was also tested.

Routine PLG phenotyping was done using agarose gel isoelectric focusing (AGIF) at pH 5-8 (Pharmalyte) as described by Dykes et al (1983) without neuraminidase. Questionable samples on infants, unusual variants and maternal exclusions were also tested after treatment with neuraminidase.

Quantitation of PLG was done by immunological means using Plasminogen QUIPlates from Helena Laboratories. The radial immunodiffusion plates measured PLG in mg/dl. A control population of 49 healthy male and female blood donors was used to obtain normal ranges and mean PLG values.

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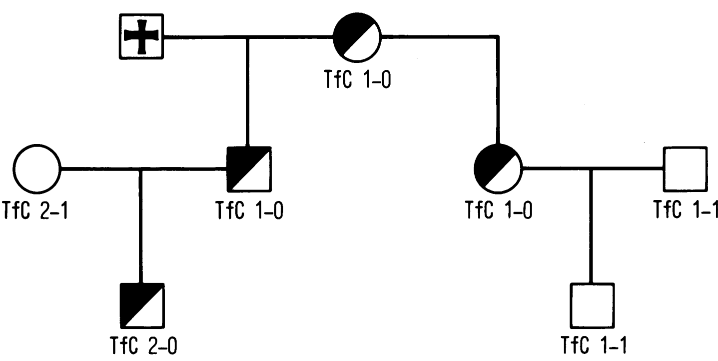
Table 1: Serum transferrin concentrations

Mean value*	573 mg/100 ml	100%
Child, Tf C2-0	150 mg/100 ml	26%
Witness, Tf C1-0	160 mg/100 ml	28%
Witness' mother, Tf C1-0	170 mg/100 ml	30%
Witness' aunt, Tf C1-0	230 mg/100 ml	40%

* N=45, SD=61,4 mg/100 ml; Range: 458-688 mg/100 ml.

Figure 1: Pedigree of the family



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Further evidence of a silent Tf allele

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Since the detection of transferrin polymorphism by Smithies in the year 1957 some cases of inherited atransferrinemia have been described in the clinical literature (Čáp et al.1968; Sakata et al.1969; Heilmeyer et al.1981; Goya et al.1972). However, only 3 well documented cases of an apparently "silent" Tf allele have been reported so far (Polesky et al.1983; Weidinger et al.1984; Lukka et al.1985).

Recently, in a criminal case of father-daughter sexual abuse (incest) in which a friend of the mother was originally involved only as a witness we observed a further Tf null allele.

24 blood-group systems (including HLA) were investigated. The paternity of the defendant was excluded in four different systems. An apparent incompatibility in the Tf system was noted between the child and the witness: the child was originally classified as Tf C2, the witness as Tf C1. In 23 other systems the witness could not be excluded as the father of this child.

The calculated plausibility of paternity for the witness (putative father) was W=99,9965% (Tf system was omitted from the calculation). The high W-value ("paternity practically proved") strongly suggested the existence of a "silent" Tf allele in the child and the putative father.

Quantitative determinations of the serum transferrin were performed employing single radial-immunodiffusion (NOR-Partigen-Transferrin, Behring). In the sera of the child and the putative father less than 50% of the normal transferrin concentration was found.

The child had 150 mg/100 ml, the putative father 160 mg/100 ml transferrin, respectively. We assumed that both are heterozygous for Tf 0 allele (see table 1).

We have been able to investigate sera of some other members of the witness' family and we found that the mother of the witness and her sister are heterozygous for Tf 0 allele, too.

The quantitative determinations of the serum transferrin concentrations of the four examples of Tf 0 allele and the mean value of 3 normal Tf phenotypes (TfC 1-1, 2-1, 2-2) are given in table 1. The pedigree of the family is given in figure 1.

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concentration. In child's serum was detected a very low concentration (Fig. 5).

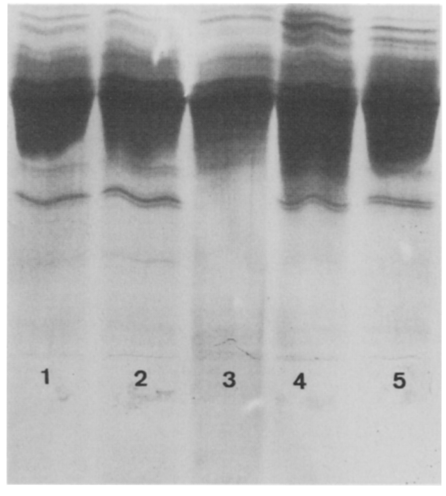

Fig. 4 – Transferrin (Tf) phenotypes: $1=C_1C_3$; 2=alleged father, TfC_1QO 3=child, TfQO 4=mother, TfC_1QO $5=C_1C_2$

Control serum – 280 mg/100 ml – 100 %

2. P. Father	– TfC_1QO	– 116 mg	– 41 %
4. Mother	– TfC_1QO	– 112 mg	– 40 %
3. Child	– TfQO	– 35 mg	– 12, 5 %

Fig. 5 – Quantitative determination of serum Tf levels by single radial immunodiffusion.

The presence of a "null" allele was then assumed in this trio. A Tf "null" allele was detected in both mother and pretense father in heterozygous states-Tf C1QO as well as in the homozygotic form in the child-TfQO.

When "null" alleles occurs in a polimorphic protein systems, difficulties may arise without a more accurate study. The presence of such alleles should be considered, in reaching a conclusion in disputed paternity.

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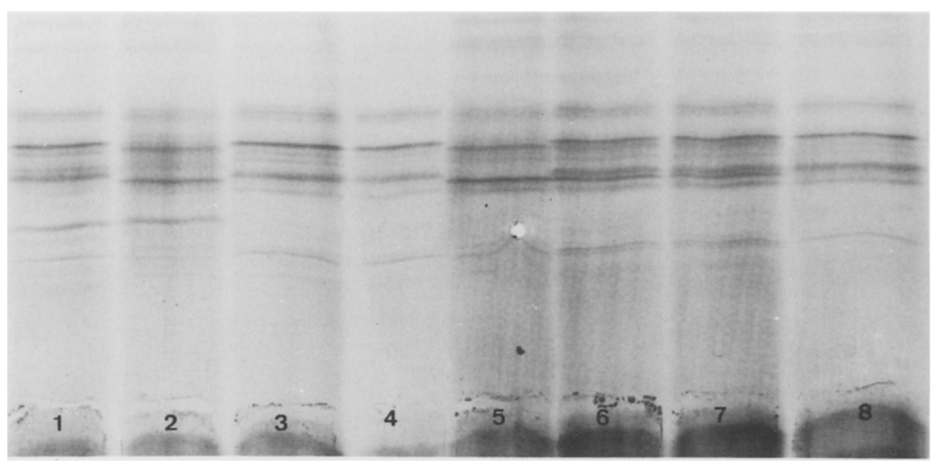

Fig. 2 - IEF of alpha-1-antitrypsin (Pi) phenotypes:
1=PiM_1S 2=PiM_2S 3=alleged father II-3, PiM_1;
4=child III-1, PiM_1(QO); 5=mother II-2, PiM_2(QO)
6=grandfather I-1, PiM_1M_2; 7=brother II-1, PiM_1M_2
8=grandmother I-2, PiM_1(QO).

	Control serum	- 275 mg/100 ml	-	100 %
Family members	II3 = PiM_1	- 259 mg	-	94 %
	II2 = M_2QO	- 144 mg	-	52 %
	III1 = M_1QO	- 79,9 mg	-	29 %
	I1 = M_1M_2	- 275 mg	-	100 %
	I2 = M_1QO	- 156 mg	-	56 %
	II1 - M_1M_2	- 269 mg	-	98 %

Fig. 3 - Quantitative determination of serum α_1-AT concentrations by single radial immunodiffusion.

In other case of paternity analysis, the child had a no detectable transferrin phenotype, whereas mother and pretense father had both C1 phenotypes, as can be seen in Figure 4.

The case was studied by immunofixation analysis with a monospecific anti-Tf-immunoglobulin. Neither other common variants were observed nor rare Tf variants were obtained in the child and his parents.

No other inconsistencies were observed in the other systems studied in this case, and there is no history of consanguinity.

Quantitative determinations of serum transferrin were made by the single radial immunodiffusion assay. In mother's and pretense father's serum was found less than 50 % of the normal transferrin

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Quantitative determinations of serum transferrin and alpha-1-antitrypsin were performed by the radial immunodiffusion method on agarose plates.

RESULTS and DISCUSSION

An apparent exclusion of maternity was found in Pi system in a Caucasian trio tested for disputed paternity (Fig. 1).The phenotypes of the trio - II2; II3; III1 - would exclude the mother. However no other contradictions to the rules of inheritance were observed in the other genetic systems (red cell antigens, serum proteins and red cell enzymes). A family study was performed and a similar apparent exclusion is also present.

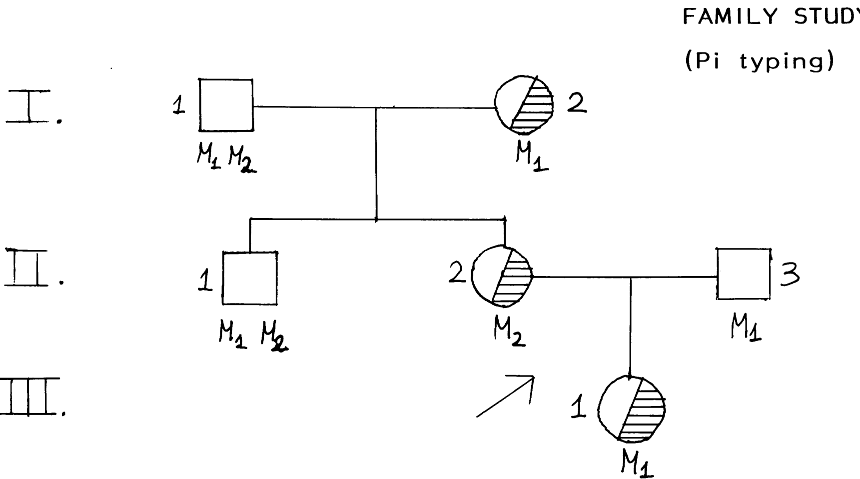

Fig. 1 - Pedigree of the family with Pi* QO carriers.
("null"); = carriers of PiQO

No other inconsistencies were observed in the other systems studied on this family.

The existence of a "null" allele was therefore, postulated in this case: The child was classified as PiM1QO, the mother as PiM2QO and the grandmother as PiM1QO. The results of alpha-1-antitrypsin testing are shown in Figure 2.

These finding were confirmed by quantitative studies.

The alpha-1-antitrypsin concentration in grandmother's, mother's and child's sera was reduced to approximately 50 % of normal.

In Figure 3 the results of serum α_1-AT concentrations are shown.

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"Silent" Alleles in Paternity Testing

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INTRODUCTION

"Null" alleles appear at almost all the genetic systems used in disputed parentage cases. So far, it seems that 0.001 value is a reasonable estimate for null alleles in most systems according to the reports of Gershowitz (1983) and Polesky et al. (1983).

Goya et al.(1972) mentioned a patient with a deficiency of transferrin in which only traces of transferrin are detected in his serum. His parents had a serum transferrin concentration about half the normal value. It presumably represents a case of familial congenital atransferrinemia. A transferrin "null" alleles has been observed by different authors (Weidinger et al. 1984; Giari et al.1985; Lukka et al. 1985). A alpha-1-antitrypsin (Pi) "null" allele in the heterozygotic form was also described by Weidinger and Cleve (1984) in a case involving a child-pretense father pair.

We found two cases with "silents" alleles in two polimorphic proteins — transferrin (Tf) and alpha-1-antitrypsin (Pi).

MATERIAL and METHODS

We used sera from disputed paternity cases routinely examined in our institute. Sera were separated and stored at -20 ºC.

For Tf subtype determination, the serum samples were diluted 1:5 with 0.5 % w/v ferrous ammonium sulphate and incubated overnight at 4 ºC.

Isoelectric focusing (IEF) was carried out in thin-layer polyacrylamide gels (5% T, 3 % C; 230X105X0,5 mm). Ampholyte concentration was 5,6% . For Pi typing, the pH range was established with a mixture of 0.6 ml of ampholyte pH 3.5-5 and 0.15 ml of ampholyte pH 4-6. Ampholine pH 5-7 was used for Tf typing. Staining of the gels was carried out with Coomassie Blue R 250.

In the case of Tf, immunofixation was made directly on the gel with monospecific antiserum against human transferrin at a dilution of 1:4 (Dakopatts).

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Phänotyp	beobachtet		erwartet	
	n	%	n	%
1	3660	73,20	3645	72,90
2-1	867	17,34	888	17,76
2	59	1,18	54	1,08
3-1	351	7,02	360	7,21
3-2	55	1,10	44	0,88
3	8	0,16	9	0,18
total	5000	100,00	5000	100,01

Genfrequenzen: $PGP^1 = 0.8538$ $\chi^2 = 0,0582298;\ 0,99 < P(df=3)$
$PGP^2 = 0.1040$ AVACH = 12,15 %
$PGP^3 = 0.0422$

Tab. 1 PGP-Phänotypen in Nordrhein-Westfalen

Die Tab.1 zeigt die Phänotyp- und Genfrequenzen aus dem westdeutschen Raum (NRW) aufgrund der Untersuchung von 5000 nichtverwandten Erwachsenen. Die isolierte Vaterschaftsausschlußchance beträgt für das PGP-System 12,15 %.

Km \ Kd	1	2-1	2	3-1	3-2	3
1	1406	153		65		
2-1	159	173	12	6	11	
2		38				
3-1	65	9		67	8	5
3-2		7	2	10	1	2
3				1		
total	1630	380	14	149	20	7

Tab. 2 2200 Mutter-Kind-Paare

Die Tab.2 gibt Auskunft über 2200 Mutter-Kind-Paare. Die Zahl der "kritischen" Mutter/Kind-Verbindungen beträgt 1663. Danach dürfte international die Mindestzahl von 2200 "kritischen" Mutter/Kind-Paaren (Nullergebnisrechnung) für die Sicherheitsgrenze von 99,8 % deutlich überschritten sein, sodaß in der Abstammungsbegutachtung einem Merkmalsausschluß volle Beweiskraft zuzumessen ist.

Die Tatsache, daß in Vaterschaftsgutachten das PGP-System rel. selten anzutreffen ist, ist offensichtlich dadurch begründet, daß die Färbemethode keine umschriebenen Enzymspots liefert und die Trennung des homozygoten Typs PGP 1-1 vom Typ PGP 3-1 bei mangelnder Übung oft nicht gelingt.

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Die Abb.4 zeigt das Ergebnis der semiquantitativen Untersuchungen der auf Hgb eingestellten Proben; in Pos.1 und 2 die Kindesmutter, in Pos.3 und 4 das Kind, jeweils mit einfacher und doppelter Dosis. In Pos.5 und 6 unverdünnte PGP 1-1 und in Pos. 7,8 und 9 unverdünnte PGP 2-2, 3-2 und 2-1 Typen.

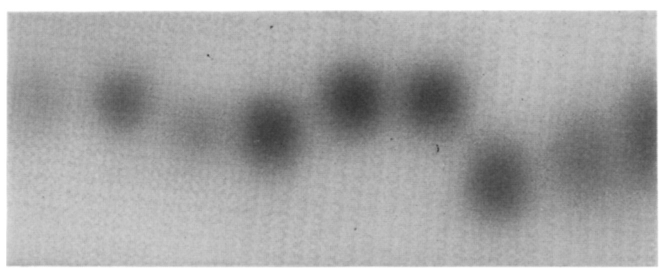

Abb. 4 Dosisuntersuchungen
Pos.1 und 2 1-0 einfach 1-0 zweifach (Mutter)
Pos.3 und 4 3-0 einfach 3-0 zweifach (Proposita)
Pos.5-9 1-1 2-2 3-2 und 2-1 Kontrollen

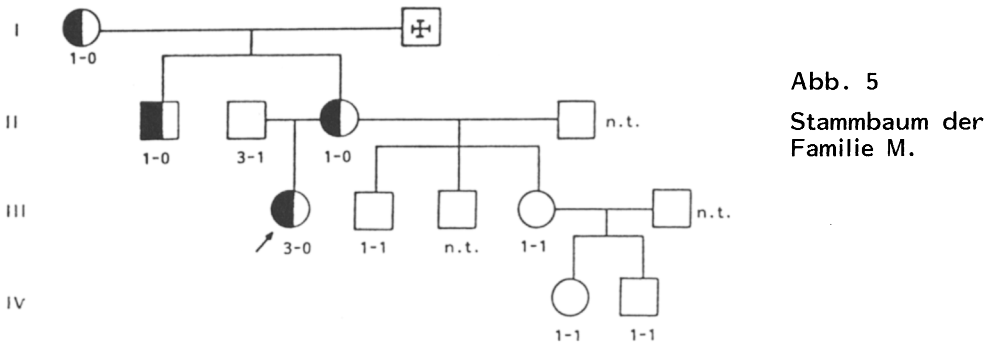

Abb. 5

Stammbaum der Familie M.

Nach unserer Kenntnis gibt es noch keine quantitative Untersuchungsmethode zur Differenzierung der phänotypischen Expression der PGP-Allele. Aufgrund der geschilderten Ergebnisse gehen wir jedoch davon aus, daß in der Familie M das "stumme Gen" PGP^{O} in drei Generationen segregiert.

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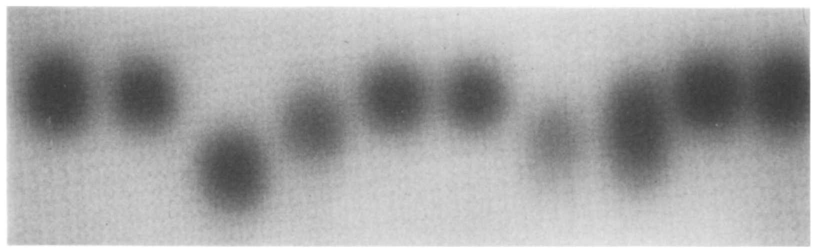

Abb.1 PGP Phänotypen im Stärkegel
von li. n. re.: PGP 1-1 1-1 2-2 3-3 1-1 1-1 3-2 2-1 1-1 1-1

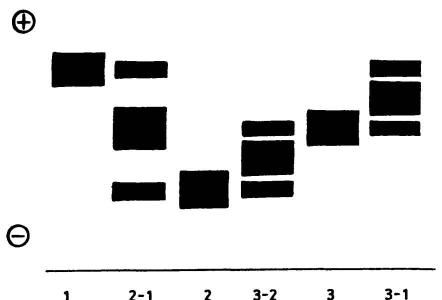

Abb. 2
Schematische Darstellung der bisher beobachteten PGP-Phänotypen (n. Barker u. Hopkinson)

Bei einem 13-jährigen deutschen Mädchen (Proposita), das anamnestisch und klinisch unauffällig war, beobachteten wir ein ungewöhnliches Zymogramm. Wie aus Pos.6 der Abb.3 ersichtlich, handelt es sich um ein PGP 3 mit sehr starker Verminderung der Enzymaktivität. In Pos.7 befindet sich eine reinerbige Kontrolle PGP 3-3. Die daraufhin durchgeführten Familienuntersuchungen ergaben bei der Kindesmutter (Pos.5) eine noch stärkere Reduktion der Aktivität des Typs PGP 1, sodaß eine scheinbare Unverträglichkeit zwischen Mutter und Kind gegeben war. Weitere schwache PGP 1-Isoenzyme konnten wir bei der Großmutter (Pos.4) und bei einem Onkel (Pos.11) feststellen. Die Verhältnisse werden durch die reinerbigen PGP 1-1 Kontrollen in Pos. 1-3 bzw. 8-10 deutlich.

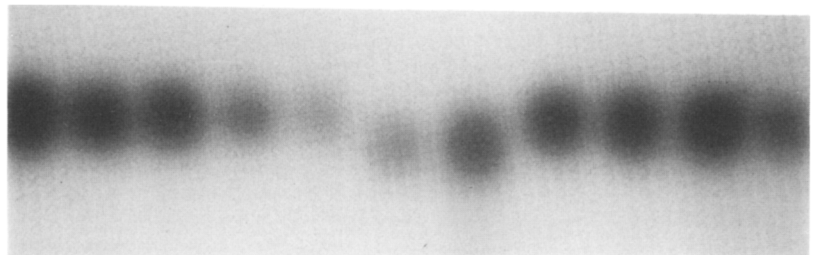

Abb. 3 PGP^{O}-Blute
Pos.1-3 und 8-10 1-1 Kontrolle
Pos.4,5 und 11 PGP 1-0 (Großmutter, Mutter, Onkel)
Pos. 6 PGP 3-0 (Proposita)

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NACHWEIS DES "STUMMEN GEN" PGP^{O} AM PHOSPHOGLYCOLAT-PHOSPHATASE-LOCUS

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Der Polymorphismus der Phosphoglycolat-Phosphatase (PGP), wie er von Barker und Hopkinson (1978) beschrieben wurde, besitzt drei Allele, PGP*1, PGP*2 und PGP*3. Nach Gal, Reeders, Waldherr und Schmidt (1986) ist der PGP-Locus auf dem Chromosom 16 (16pter-p11) eng mit dem Gen für die polycystische Nierenerkrankung (PKD1) gekoppelt.

METHODE

(n. Barker und Hopkinson, 1978 mod. von Martin, 1981)

Gelherstellung: 50 g Toronto-Stärke (Biomol 07378) wird in 30 ml Brückenpuffer und 140 ml Aqua dest. suspendiert, in 250 ml kochendem Aqua dest. eingerührt und auf die Glasplatte (18-22 cm) gegossen.
Brückenpuffer (pH 7,2): 30,0g Tris 20,0g Maleinsäure, 4,0g NaOH ad 2000 ml Aqua dest.

Probenauftrag: 4 cm von der Kathode entfernt werden 2 Schlitzreihen im Abstand von ca. 8 cm gestanzt und 10x8 mm große hämolysatgetränkte Filterpapiere eingelegt.

Elektrophorese: 15 Std. bei 120 V, 30mA in der Desaphor-Kammer bei 4°C.

Färbung: Das Gel wird aufgeschnitten, die untere Hälfte auf eine Glasplatte gelegt und das Overlay daraufgegossen. Es folgt eine Inkubation von ca. 3,5 Std. bei 37°C, dann wird das Overlay entfernt und die Färbelösung auf das Gel verteilt; weitere 30 Min. bei 37°C inkubiert. Die überschüssige Färbelösung wird mit Zellstoff abgenommen.
Overlay (pro Reihe): 150 mg Agarose Seakem ME (FMC 62626), 15 ml Entwicklerpuffer, 50 mg Glycolat-2-Phosphat (BM 127167).
Entwicklerpuffer: 6,0g Tris, 05,g MgCl2x6 H_2O, 0,2g NaN3 ad 500 ml Aqua dest. (mit 10% HCl pH 7,3 einstellen).
Färbelösung: 60 mg Ammoniummolybdat in 5 ml 4 N H2S04 lösen, 75 mg Ascorbinsäure zugeben.

ERGEBNISSE UND DISKUSSION

Die Abb.1 zeigt die Trennung von 5 PGP-Phänotypen im Stärkegel und die Abb.2 die schematische Darstellung aller bisher beobachteten Phänotypen. Die einzige Beobachtung einer Variante stammt von Henke et al. (1985) bei einer indonesischen Frau und ihrem Kind. Die Variante wurde vorläufig mit PGP 1-Sumatra bezeichnet, wobei das variante Isoenzym anodisch von dem des normalen PGP 1 gelegen ist.

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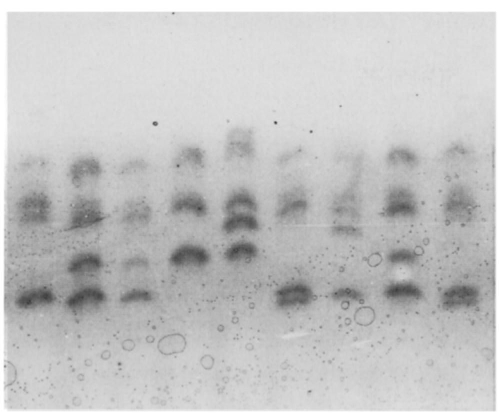

Fig. 2. Photograph of PGM_1 subtypes determined by IEF on 0.5mm polyacrylamide gel /pH 5-7/. Samples from left to right: 1+, 1+2-, putative father 1+2-, child 2-, mother 2-2+, 1+1-, 1+2+, 1+2-, 1+1- /second case/

Polyacrylamide gel isoelectric focusing revealed the existance of weak bands in the proband /first case/ and in putative father /second case/ patterns corresponding to isoenzyme 2- /Fig. 1 and Fig. 2/. In these findings the putative fathers were not excluded.

The results suggest that not only typing by starch gel electrophoresis but also subtyping by isoelectric focusing ahould be performed, especially in forensic investigations auch as testing for paternity.

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Proband: O, MSs, Rh:CCDee, kk, P_1+, Hp 2-2, Gm 1, -2, Gc 1, C3 FS, AcP B, EsD 2-1, GLO 2-1, PGM_1 1, HLA-A3, B14, 44/12/, w4, w6, Cw7

Mother of proband: O, MNSS, Rh:CCDee, kk, P_1-, Hp 2-2,Gm 1, -2, Gc 1, C3 FS, AcP B, EsD 2, GLO 1, PGM_1 1, HLA-A2, 3, B14, w62/15/, w6, Cw3

Second case

Putative father: O, MNSs, Rh:CcDee, kk P_1+, Hp 1-1, Gm 1, 2, Gc 1, C3 FS, AcP B, EsD 1, GLO 2, PGM_1 1, HLA-A2, 24/9/, Bw 56/22/, w62/15/, w6, Cw3

Proband: A_2, MNSs, Rh: CcDee, kk, P_1+, Hp 1-1, Gm 1, 2, Gc 2-1, C3 S, AcP B, EsD 1, GLO 2-1, PGM_1 2, HLA-A11, 24/9/, B13, w56/22/, w4, w6, Cw3, w6

Mother of proband: A_2, MSs, Rh:CcDee, kk, P_1+, Hp 1-1, Gm 1, -2, Gc 2-1, C3 FS, AcP BA, EsD 1, GLO 2-1, PGM_1 2, HLA-A1, 11, B13, w4, Cw6

In these findings the putative fathers ought to be excluded. For this reason the PGM_1 subtypes were examined by means of IEF. The results were as follows:

	first case	second case
putative father	2-	1+ 2-
proband	1+ 2-	2-
mother of proband	1+	2- 2+

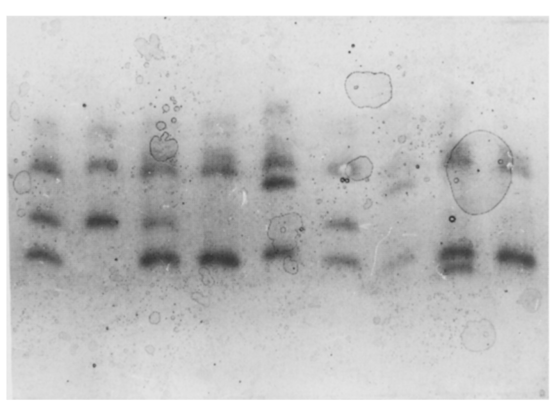

Fig. 1. Photograph of PGM_1 subtypes determined by IEF on 0.5mm polyacrylamide gel /pH 5-7/. Samples from left to right: 1+2-, putative father 2-, child 1+2-, mother 1+, 1+2+, 1+2-, 1+2+, 1+1-, 1+ /first case/

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Silent Alleles

Contrary Homozygozity of The PGM_1 System in Disputed Paternity

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INTRODUCTION

Since the original discovery of the polymorphism of phosphoglucomutase in human red cells /Spencer et al. 1964/, a number of investigators and population studies of the enzyme have been described. Isoelectric focusing either in polyacrylamide or agarose gels has become the method of choice for subtyping of the PGM isoenzymes determined by the PGM_1 locus /Bark et al. 1976; Kühnl et al. 1977; Sutton and Burgess 1978/.

During the determination of blood groups in forensic cases of disputed paternity in the last year 1986 in two cases an incopatibility between a putative father and a child in PGM_1 system was observed.

METHODS

All samples of blood have been investigated routinely in following genetic marker systems such as: ABO, Rh, MNSs, Kk, P_1, Hp, Gm 1, 2, Gc, C3, AcP, PGM_1, EsD, GLO and HLA-ABC. The PGM_1 phenotypes were tested by use starch gel electrophoresis and by means of isoelectric focusing /IEF/.

RESULTS

The following results was observed:

First case

Putative father: O, MSs, Rh:CcDee, kk, P_1+, Hp 2-1, Gm 1, 2,
Gc 1, C3 FS, AcP B, EsD 1, GLO 2, PGM_1 2,
HLA-A3, 11, B35, 44/12/ w4, w6, Cw4, w7

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EINE METHODE ZUR SCHNELLEN UND KOSTENGÜNSTIGEN ANALYSE GERINGER PROBENMENGEN MITTELS IEF IN DÜNNEN GELEN

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Es wird über praktische Erfahrungen mit der isoelektrischen Fokussierung (IEF) von Serumproteinen an ultradünnen Polyacrylamidgelen eigener Fertigung im "Diapositiv-Format" mit Hilfe des "Phast-Systems[R]" berichtet.

Das methodische Vorgehen wird genau beschrieben:
Die praktische Herstellung der Gele, die Anfärbung, Temperatur und die elektrischen Grössen wie Spannung, Stromstärke und Volt-Ampere-Stunden. Der Vorteil der Methode liegt in der kleinen Probenmenge, dem geringem Materialverbrauch und damit Kostengünstigkeit. Die Ergebnisse liegen rasch vor (unter 1 Stunde) und die Handhabung ist vereinfacht.

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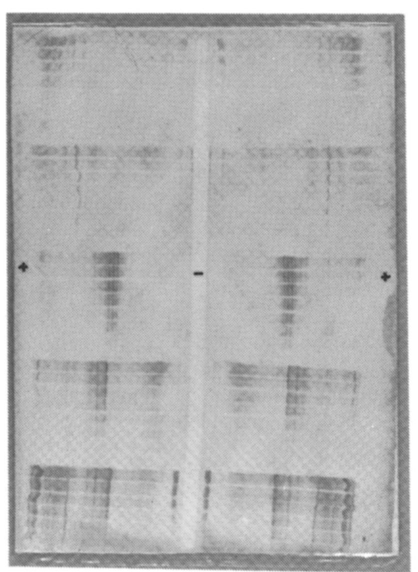

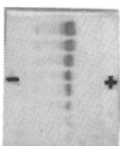

Fig. 2. Large Scale IEF gel (to the left) 200 x 250 x 0.5 mm, a three electrode system separating 60 samples on one gel, Ampholine pH gradient 3.5 - 9.5. To the right a mini gel 35 x 45 mm, a dilution series of hemoglobin, Ampholine pH range 3.5 - 9.5.

This Film Remover makes it possible to remove one part of the gel for electroblotting while the rest is left on the support film to facilitate standard staining procedures, without any risk of gel swelling or cracking.

1. Swedish patent 8605190-1, other patents pending.

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Electrophoretic Transfer of Proteins from Electrofocusing Gels Cast on a Plastic Support Film

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INTRODUCTION

The trend in most electrophoretic techniques is towards using thin or ultra-thin gels (0.1-0.5 mm) for increased band sharpness and faster separations.

Casting the gel on a plastic support film facilitates the handling of the gel and eliminates the risk of alteration in gel size due to swelling during the steps involved, especially when pore gradient gels are used.

However, the plastic support film has to be removed before blotting, to enable the current to pass through the gel during this step.

The device shown here has been designed to facilitate easy removal of the gel from the plastic support regardless of gel or film size and thickness.

MATERIALS AND METHODS

The Film Remover device (LKB 2117-225) was evaluated with polyacrylamide gels containing pH 3.5 - 9.5 Ampholine (LKB 1818-101). IEF was performed on an LKB 2117 Multiphor II System and the electroblotting step was carried out with the LKB Multiphor II NovaBlot using a continuous buffer system. Tris (39 mM) Glycine (48 mM) 0.0375% SDS and 20% Methanol.

CONCLUSIONS

The Film Remover device shown here conveniently removes the plastic support film from lab-cast and pre-cast gels.

The gel sizes that can be applied are in the range 200 x 250 mm down to 35 x 45 mm; and the gel thicknesses from 0.1 mm up to 1-5 mm.

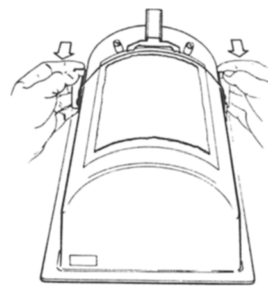

Fig. 1. Schematic drawing showing the LKB 2117-225 Film Remover device (Ref. 1).

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DISCUSSION

Up to now, two major views have been advanced to explain the genetics of PGA: a locus system (Samloff and Townes 1970; Weitkamp and Townes 1975; Taggart et al. 1979; Frants et al. 1984) and a multilocus system (Korsnes and Gedde-Dahl 1980). Our study has provided evidence that one component of PGA, fraction V, is polymorphic with two alleles, F and S, at a single locus. Similar observation have not been reported by others previously, as the present results were obtained using a new combination technique involving both IEF and immunoblotting with PGA-specific antibody, which facilitated high resolution of the individual urinary pepsinogen isozymogens. According to our classification method based on the differences in pI values, rather than on the intensity levels of isozymogens, phenotype S is clearly distinguishable from F on the gel. Moreover, instead of a proteolytic activity test for the isozymogen, an immunoblot technique with specific anti-PGA antibody was used in this study. This immunological detection method facilitated discrimination of PGA isozymogens from other proteolytic enzymes like PGC and slow-moving protease (SMP).

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Table 1. Distribution of phenotypes and allele frequencies of human PGA V among unrelated Japanese

Phenotypes	Observed number	Observed frequency	Expected number	χ^2
S	224	0.868	223.2	0.0029
FS	32	0.124	33.5	0.0672
F	2	0.008	1.3	0.3769
Total	258	1.000	258.0	0.4470

Allele frequencies: PGA V*S=0.930, PGA V*F=0.070; d.f.=1; 0.7>p>0.5

Table 2. Segregation of PGA V phenotypes

Mating	Number of families	Number of children	Children's phenotypes F	Children's phenotypes FS	Children's phenotypes S
F x FS	1	2		2	
FS x S	3	5		3	2
S x S	34	52			52
FS x N.T.[a]	1	3		2	1
S x N.T.[a]	1	3			3
Total	40	65	0	7	58

[a]Not tested because one parent was unavailable for this study.

Inheritance of PGA V F and PGA V S Bands

The family materials were matings with three PGA V-typed children. The inheritance of the isozymogen pattern was investigated by listing the numbers and phenotype categories of the offspring observed for each different type with regard to the presence or absence of each PGA band. Three of the theoretical six types of mating were observed among the three phenotypes.

S behaves as a Mendelian dominant, this being strongly supported by the absence of F among the 52 offspring of 34 S x S matings. The F band is likewise a clear Mendelian dominant, as shown in Table 2, since in an F x FS mating, each of the two types of children have received only one of the corresponding characteristics from each parent, PGA V*F from the father and PGA V*S from the mother, and therefore have no intense S band. We may conclude from the above observation that the presence of an F or S band is governed by at least one pair of alleles and by a structural gene dose effect.

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freeze-dried urinary sample, corresponding to about 100 times the concentration of the original urine, were used for IEF analysis. After IEF, PGA isozymogens on IEF gels were detected by immunoblotting using anti-PGA antibody (Yasuda et al. 1987).

RESULTS

Phenotypic Variation and Nomenclature of PGA Isozymogens

The detection method involving IEF followed by blotting transfer to a Durapore (Millipore) strip and immune binding showed that PGA was generally separable into five fractions on the gel between pI 3 and 4, as shown in Fig. 1, and these fractions were named I to V in order of decreasing anodal mobility.

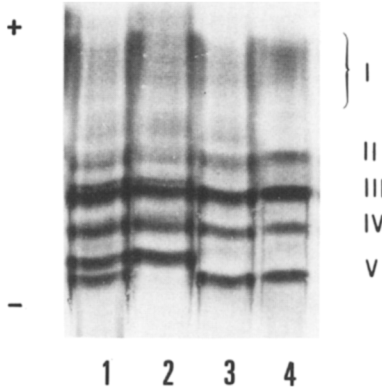

Fig. 1. Isoelectric focusing of urinary samples in polyacrylamide gel and visualization of uropepsinogen (PGA) by immune blotting with specific antibody. Samples were (1) FS, (2) F, (3) S, and (4) S.

Several subjects showed an additional band between IV and V, being nearer to V, as shown in Fig. 1. At this point it is convenient to introduce a new form of nomenclature: this band will be termed the PGA V F band, indicating that it is a fast-moving band, while the normal V band will be termed PGA V S, a slow-moving band. The F band was found to be present in 34 out of 258 Japanese subjects, while the S band was absent in 2 out of the 34 subjects having the intense F band (Table 1). Phenotype FS, containing both F and S bands, corresponds to a phenotype which is a mixture of two types, F and S. The distinct nature of the three discrete phenotypes in the V fraction remained apparent; the F band without the S band exhibited a higher intensity than the F band with S. Similarly, the S band without F had a higher intensity than S with F.

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Genetic Polymorphism of Urinary Pepsinogen (PGA) Detected by Immunoblotting

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INTRODUCTION

Pepsinogen is classified into two immunochemically distinct groups, PGA and PGC. PGA is detected in serum and urine as uropepsinogen, and while PGC is present in serum and seminal fluid. Genetic models of PGA have been proposed by several investigators (Samloff and Townes 1970; Taggart et al. 1979; Korsnes and Gedde-Dahl 1980; Frants et al. 1984), who have used all the proteolytic properties of acid-activated PGA for the detection of the isozymogens on agar or polyacrylamide gels. However, the complexities of these techniques have yielded a number of conflicting results, leading to various different genetic models.

In the present work, we describe a new technique utilizing polyacrylamide gel isoelectric focusing (IEF) followed by immunoblotting with anti-PGA antibody, which provides a better band resolution, and propose that a number of the uropepsinogen isozymogens with different pI values can be explained by the presence of one autosomal locus with two alleles (Kishi and Yasuda 1987).

MATERIALS AND METHODS

Purification of PGA for Immunization

PGA for immunization was purified from human urine mainly using the method described by Minamiura et al. (1984). Rabbit antisera against purified PGA were obtained as described previously (Kishi and Iseki 1983). A total of 4 mg of the purified PGA was injected into a rabbit.

Detection of PGA Isozymogens on IEF gels by Immunoblotting

The urine samples for IEF were concentrated by ultrafiltration, dialyzed against 0.1% glycine and then lyophilized according to the method described in our previous paper (Kishi et al. 1985). Five-microliter aliquots of a 0.5-1.0% solution of

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gives efficient and clear separation.
The formal genetic data confirm an autosomal codominant transmission of the alleles. The frequencies in Southwestern Germany are:
A2HS*1=0.631, A2HS*2=0.363, A2HS*V=0.006.

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tions of the original gene products. Method 1 as well as method 2 reveal series of bands, the distance between the single bands being fairly small. Clear separation of the major gene products a2hs*1 and a2hs*2 was improved using desialyzed samples and IEF with 2.5 M urea in the pH-range 5-6 (method 3). In combination with Western-Blotting this method seems to give the best results. Homozygotes A2HS 1 and A2HS 2 each show a single main band with different IEP. These two main bands can consequently be observed also in heterozygote individuals. So the results of desialyzation demonstrate that the complex banding patterns, seem to be caused by different sialyzation status of the A2HS gene products.

Formal genetics

Tab.1. Segregation of the A2HS phenotypes in 124 families (Klett 1985).

mating type	number of fam.	ch.	phenotypes of children 1	2-1	2	4-1	4-2	x^2	df
1x1	25	84	84						
1x2-1	39	108	44	64				3.704	1
1x2	14	40		40					
2x1-2x1	22	66	22	32	12			3.091	2
2x2-1	20	57		33	24			1.421	1
2x2	2	7			7				
4-1x2-1	2	6	2	1		1	2	0.667	3
Total	124	368	152	170	43	1	2	8.883	7

Tab.1 presents the segregation analyzes, performed in 124 families including 368 children from Southwestern Germany. The results, showed above, support the hypothesis "3 alleles (a2hs*1, a2hs*2, a2hs*4) at an autosomal locus" (x^2 = 8.883, 0.70-P-0.80, df=7).

Population genetics

Population studies were conducted on 249 unrelated individuals from Southwestern Germany. The allele frequencies in this sample were determined to be : A2HS*1=0.631, A2HS*2=0.363, A2HS*V=0.006 (V=A2HS*4 + A2HS*Var.).

SUMMARY

The genetic polymorphism of A2HS was investigated by different methods. The technique, using desialyzed plasma, Pharmalytes in the pH-range 5-6 and 2.5 M urea, followed by Western-Blotting,

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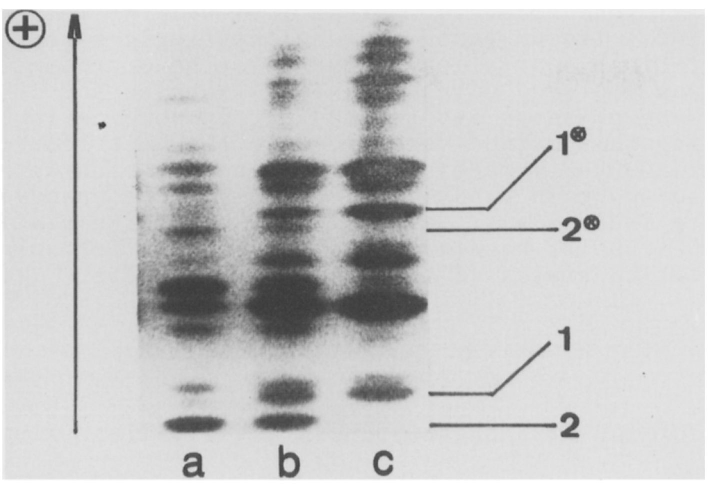

Fig.1. A2HS profiles in native samples, analyzed by IEF with Immobilines in pH 4-5, detected by immunofixation:
a = A2HS : 2
b = A2HS : 2-1
c = A2HS : 1

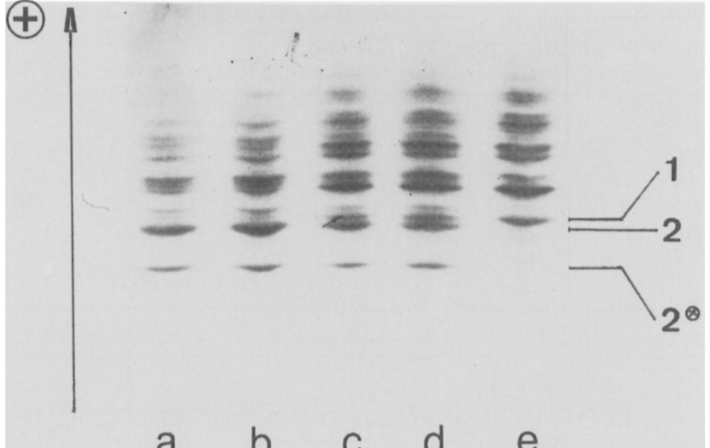

Fig.2. A2HS profiles in native samples, analyzed by IEF in 2.5 M urea with carrier ampholytes in pH 4-6, detected by Western-Blotting and EIA :
a,b = A2HS : 2
c,d = A2HS : 2-1
e = A2HS : 1

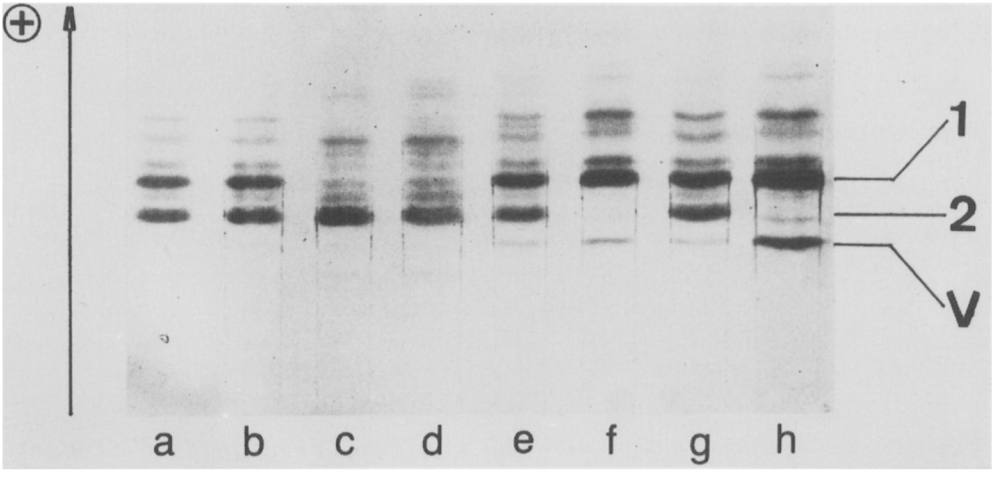

Fig.3. A2HS profiles in desialyzed samples, analyzed by IEF in 2.5M urea with Pharmalyte in pH 5-6, detected by Western-Blotting and EIA : a,b,e,g=A2HS:2-1; c,d=A2HS:2; f=A2HS:1; h=A2HS:1-V

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print contact 1 h at 37 deg.C, CAF washed overnight, stained with 0.115% Coomassie Brilliant Blue G 250 (Serva) in destainig solution (25% ethanol, 8% glacial acetic acid in A. bidest.).

2. IEF with carrier ampholytes in the pH-range 4-6 in 2.5 M urea, using native samples, detected by Western-Blotting.

Polyacrylamide gel: 2.7 ml 28% Acrylamide (LKB), 1.2 ml 2% N,N'-Methylenbisacrylamide (LKB), 0.2 ml ampholytes ph 3.5 - 10, 0.8 ml ampholytes ph 4-6, 2.5 ml A. bidest., 2.5 M urea, 0.1 ml 3% TEMED (Serva), 1.0 ml 5% APS (Serva). Polymerization overnight at room temperature.
Focusing: Ultraphor with 10 deg.C temp. of cooling water, anolyte: 1M H PO , catholyte: 1M NaOH; 10 ul of undiluted plasma were placed on the gel surface with 0.5x0.5 filter papers (LKB) 1.5 cm from the cathodal end for 45', prefocusing without samples with 1500 V, 25 mA, 3 W for 30', with 1500 V, 25 mA, 5 W for 15', focusing 3 hrs.
Detection: Western-Blotting and EIA, passive transfer on Nitrocellulose Membrane NCM (Schleicher/Schüll, 0.45 um) for 40' at room temp., NCM washed in 0.05% Tween 20 (Merck) in PBS and blocked with 5% Albumin (Sigma) in PBS, reaction with the 1.antibody (rabbit anti-human alpha-2-HS-glycoprotein (Behring), 1:200 in 1% Albumin) and the 2. antibody (anti-rabbit immunoglobulin conjugated peroxidase (ATAB), 1:1000 in 1% Albumin) each for 1h at room temperature, visualization was performed by O-Toluidin dependent POD-reaction.

3. IEF with Pharmalyte in the pH-range 5-6 in 2.5 M urea, using desialyzed samples, detected by Western-Blotting.

This method, previously described by Umetsu et al. (1986), was modified by us concerning the following parameters. We changed the sample preparation, the running conditions, the anolyte solution and omitted glycerol for preparing the gel.
Polyacrylamide gel composition was the same as described in method 2 with the exception of the carrier ampholytes: 1 ml Pharmalyte pH 5-6, electrolyte solutions, running conditions and detection were as described above (method 2).
Enzyme treatement: 10 ul plasma with 20 ul neuraminidase (1mg/ml, Boehringer) were incubated at 37 deg.C in a moist chamber about 24 hrs, 9 ul of each sample were applied with 0.5x0.5 cm filter papers (LKB) at a distance of 2 cm from the cathode for 45'.

RESULTS AND DISCUSSION

Comparison of the different techniques shows, that separation of native samples by immobilines and by carrier ampholytes reveals series of A2HS bands. Detection of the different gene products seems to be best in the cathodal part of the proteinogram. The additional banding-patterns, designated by a star, are conforma-

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Polymorphism of AHS-Glycoprotein: Comparison of different IEF and detection techniques

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INTRODUCTION

The human plasma protein alpha-2-HS-glycoprotein (A2HS, Schultze et al. 1962) was first described separately by Heremans (1960) and Schmid and Bürgi (1961). A2HS consists of one polypeptide chain with a molecular weight of 49000 D. The content of carbohydrate amounts to 13.4% (Schmid/Bürgi 1961) and of sialic acids to 4.1% (Putnam 1975). Lewis/Andre 1978).
A genetic polymophism of this protein was reported by Cox and Andrews (1983) with the existence of three alleles (A2HS*1,2 and 3) at an autosomal locus. In 1984 Umetsu et al. and Weidinger reported the presence of further rare alleles. Eiberg et al. assigned in 1983 the A2HS locus to human chromosom no.3. Our aim was to present a routine method, giving a clear separation of all different gene products and to give results on family and population studies of Southwestern Germany.

MATERIALS AND METHODS

Blood was obtained from healthy donors and their families. EDTA-plasma was stored at -30 deg.C until used. The following different techniques were examined. IEF was always run in 260x125x 0.5 mm polyacrylamide gels.

1. IEF with immobilines in the pH-range 4-5, using native samples, detected by immunofixation (Klett 1985).

Polyacrylamide gel: 2.5 ml 29.1% Acrylamide (LKB)/ 0.9% N,N'-Methylenbisacrylamide (LKB), 0.751 ml immobilines pK 4.6 (LKB), 0.371 ml immobilines pK 9.3 (LKB), 9.278 ml A. bidest., 2.1 ml 87% glycerol (Merck), 0.130 ml 10% TEMED (Serva), 0.060 ml 10% APS (Serva). Polymerization at 50 deg.C for 60'.
Focusing: Ultraphor with 10 deg.C temp. of cooling water, anolyte: 0.025 M asparaginic acid, catholyte: 0.025 M glutamic acid, 15 ul of undiluted plasma were applied on 1.0x0.5 cm filter papers (LKB) 2 cm from the cathodal end for 6 hrs, running conditions: 5000 V,
25 mA, 5 W for 6 hrs and 2500 V, 25 mA, 5 W for further 14 hrs.
Detection: Immunofixation with anti-human alpha-2-HS-glycoprotein (Behring, 1:1 in physiol. saline) in CAF (Schleicher/Schüll),

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very small and therefore the number of persons limited (20 out of 5 families), there is a significant difference to other german population samples. This was not observed for the other systems tested except HP.

Table 1. A2HS phenotype and allele frequencies in the region of Ulm (Ostwürttemberg, Schwaben, Oberschwaben), 368 unrelated germans.

phenotype	n obs.	%	n exp.	%	
1	179	48.64	174.6	47.46	A2HS* 1 : 0.6889
2-1	144	39.13	152.9	41.56	A2HS* 2 : 0.3016
2	38	10.33	33.5	9.11	A2HS* Var 0.0095
1-Var	5	1.36	4.8	1.30	
2-Var	2	0.54	2.1	0.57	$\Sigma \chi^2 = 1.29$
Var	0	0	0.03	0.01	

Table 2. A2HS phenotype frequencies in an isolate population sample from the region of Ulm ("Steinheimer Wald")

phenotype	n obs.	%	% exp.(from table 1)
1	16	80.0	47.5
2-1	4	20.0	41.6
others	0	0	10.9

SUMMARY

A2HS proved to be of great value as additional system in parentage testing (with the exclusion chance of 17.8% according to Weidinger (1986)) as well as for other problems. With the reliable and reproducible separation method given here, the system should now be generally included in the standard expertises as it was already suggested.

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Segregation and linkage data

Though Eiberg (1983) reported close linkage of TF and A2HS, the analysis of a large pedigree (kindly performed by Dr. T.F. Wienker, University of Freiburg, FRG) does not show convincing values in favor of close linkage, $\zeta=0.093$ at $\theta=0.23$. One of the three informative pedigrees (figs. 2 A,B,C) shows an crossover (no sign of illegitimacy in 27 systems including HLA - candidates for a intrafamiliar bone marrow transplantation). Neither the segregation data of the pedigrees nor the other families investigated revealed a deviation from the assumed autosomal codominant inheritance nor did we observe incompatible mother-child pairs or single contrary homozygosity exclusions in about 100 cases of disputed paternity.

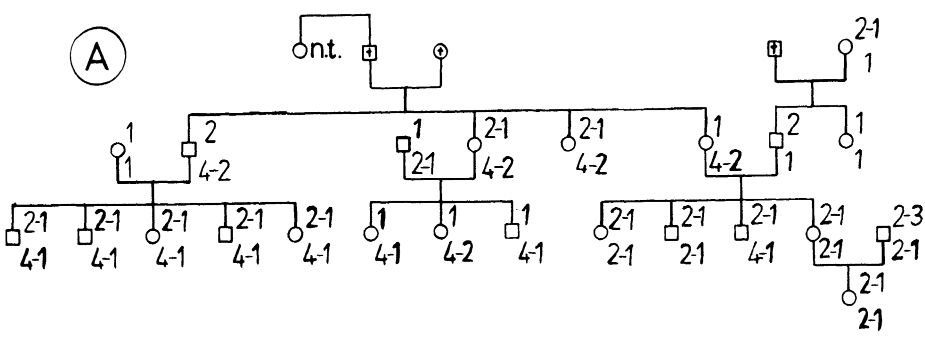

Fig. 2. A. 4-generations pedigree from a family originating from the surroundings of Stuttgart. Two persons in generation 1 come from the same little village, thus it can be assumed, that they both carried the A2HS*4. B. In this family a crossover has to be assumed. Numbers above the person's symbols refer to the TF-C subtype, those below to A2HS. C. Same as in B.

Phenotype and allele frequencies

Data from the south german population sample from the region of Ulm (Oberschwaben, Schwaben, Ostwürttemberg) are shown in table 1, they fit well with other population samples from Germany and other caucasian populations, 0.64-0.66 for A2HS*1 and 0.32-0.34 for A2HS*2. Three rare variants could be observed, most frequently A2HS*4. Data from an isolate population from our region ("Steinheimer Wald") are shown in table 2. Though the isolate is

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Running conditions: Anolyt 10 mM glutamic acid, catholyt 10 mM NaOH; coolant held at 4 °C; 3000 V, 2 mA, 5 W (maximum settings) over night (16-18 hrs.) no prerun.

Print immunofixation

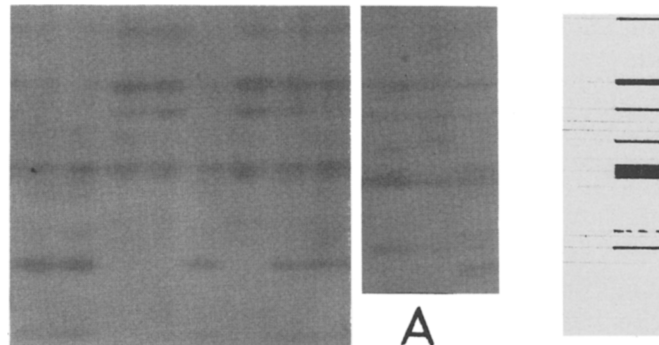
Material: The antiserum is not commercially available (in Germany), but on request from the research department of Behring(Höchst) / Calbiochem via their regional distributors. The CAF membrane was from Sartorius, type 112oo, 57 x 145 or 80 x 150 mm.

Immunofixation: The membrane is soaked with saline, excess moisture removed and the membrane carefully (with no air bubbles trapped) laid on the separation gel, from 1 cm beside the application area towards the anode. 4 µl/ cm² of the undiluted antiserum are immediately distributed on the wet membrane with a Drygalski spatula. After 20 min incubation in the moist chamber at 37°C the membrane is removed, washed 3 x 10 min. in saline and stained with coomassie blue as usual. Destaining was done under control before the membrane was transferred to the storage bath (10% acetic acid with 2% glycerol), which was found to result in a more evenly stained membrane.

RESULTS AND DISCUSSION

Separation

As shown in figs. 1A and 1B, the immobilized gradient enlarges the A2HS-region to a distance of about 5 cm, including the anodal band of A2HS 4 and the most cathodal band of A2HS 2. The resolution allows to confirm a certain phenotype on at least three iso-pH levels in a reliable and ampholyte-batch independent way. Simultaneous typing of GC and PI is, in our experience, better to be performed on a separate (part of the) gel to avoid difficulties due to the antiserum background.



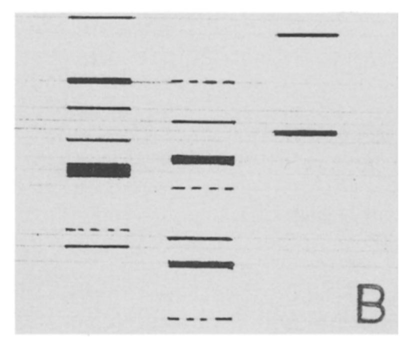


Fig. 1. A: Photographic representation of A2HS phenotypes. Lanes 1,2,5: A2HS 2; lanes 3,4,6,9: A2HS 1; lanes 7,8,11: A2HS 2-1; lane 10: A2HS 4-1. B: Schematic presentation of the bands characteristic for alleles 1,2, and 4 (from left to right, the anode is at top)

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α_2-HS-Glycoprotein (A2HS): Improved Phenotyping by Focusing in an Immobilized pH-Gradient. Allele Frequencies in a South German Population Sample and Linkage Data from a Large Pedigree

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INTRODUCTION

The use of A2HS as an additional polymorphic marker in paternity testing and related forensic applications is not yet introduced in the routine expertises of all laboratories. One of several causes is the problem of antiserum supply, another the poor and batch-dependent resolution of the specific bands by separation with commercial carrier ampholytes according to previously described procedures (Weidinger, 1986; Mendner and Kühnl, 1986). Thus we developped an appropriate immobilized pH-gradient, which allows an improved and better reproducible separation.

METHODS

Separation data

Gel dimensions: 150 x 120 x 0.5 mm

Casting chamber: Silicon rubber U-frame, Mylar and Gel-Fix sheets (LKB, SERVA) between glass plates.

Gradient mixer: 2 x 7 ml, from Hölzel, Dorfen, FRG

Gel composition: Acidic dense solution: 290 µl Immobiline pK 4.6, 100 µl pK 9.3, 1600 µl 87% glycerol, 1500 µl water; basic light solution: 250 µl pK 4.6 230 µl pK 6.2, 3100 µl water; either solution 1250 µl acrylamide 20 T 3 C. pH was 4.2 and 5.15 before addition of catalysts (3.75 µl TEMED and 10 µl 10% persulfate to 4500 µl in each chamber.

Casting and gel processing: Gels were cast without pumping according to procedures suggested by LKB (Görg, 1983), left in place to polymerize for 15 min. at room temperature, then polymerization was completed at 50°C for 1 hour. 3 washes with water,1 with 2% glycerol, each about 10 min, removed unpolymerized material. After complete drying at 50°C, the gels were stored frozen until use. Rehydration was allowed for at least 1 hour with 20% glycerol containing 2 mM Tris-base in the casting chamber.

Samples: Serum or EDTA-plasma, 4 µl applied on 4x7 mm filter papers 1-1.5 cm beside the cathodal strip

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Table 1. Segregation of the children's phenotypes in 159 families with 479 children.

mating type	number of fam.	ch.	phenotypes of children F	FS	S
F x F	21	62	62	-	-
F x FS	54	159	81	78	-
			79,5	79,5	
F x S	18	54	-	54	-
FS x FS	44	133	38	66	29
			33,25	66,5	33,25
FS x S	18	55	-	29	26
				27,5	27,5
S x S	4	16	-	-	16
Total	159	479	181	227	71

Population genetics

The population sample comprises of 336 unrelated individuals from southwestern Germany. Table 2 summarizes the observed distribution of the phenotypes and the calculated allele frequencies.

Table 2. Distribution of ORM1 phenotypes and ORM1 allele frequencies in a population sample from southwestern Germany.

Phenotypes	N	Alleles	frequency
ORM1 F	120	ORM1*F	0.609
FS	168	ORM1*S	0.388
S	46	ORM1*V	0.003
FS1	1		
SS2	1	(ORM1*V = ORM1*S1 + ORM1*S2)	
Total	336		

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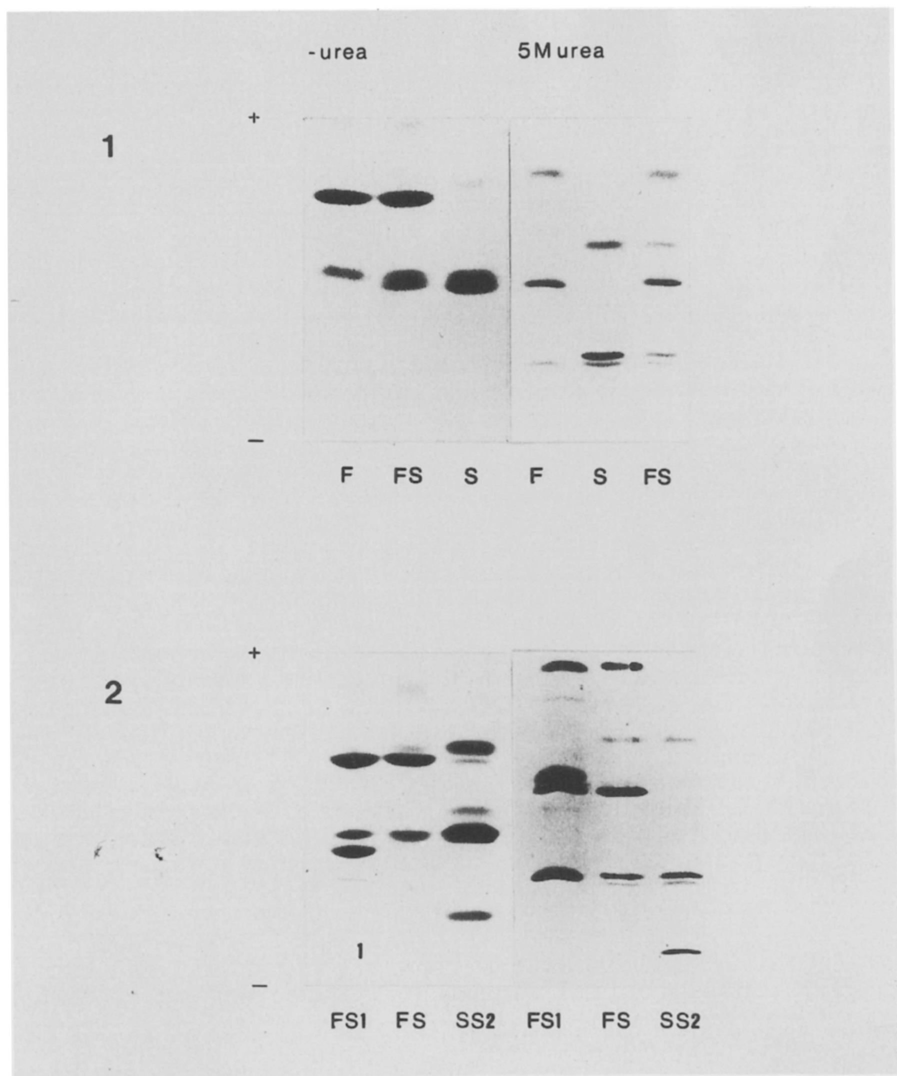

Fig. 1. Common (1) and rare (2) ORM-phenotypes after PAGIEF in the pH-range 4.5 - 5.4 in presence or absence of 5 M urea. Detection by immunofixation.

Formal genetics

Table 1 presents the segregation of the children's phenotypes in 159 families with 479 children from southwestern Germany.

The results of the segregation analysis are in agreement with the formal hypothesis "2 common alleles ORM1*F and ORM1*S at an autosomal locus ORM1". Observed and expected values of the children's phenotypes differ only by chance (X^2=1.447,df=4, 0.80-P-0.85). Since investigations at the DNA-level (Dente et al.1985) revealed the existence of two ORM loci, we preferred to designate the alleles ORM1*F and ORM1*S respectively.

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Detection: Immunofixation with anti-human α1-Glycoprotein (Sigma, 1 : 4 in 0,9% saline) in CAF (Schleicher & Schüll) with 10' print contact. Foils were washed overnight in 0,9% saline and stained with 0,115% w/v Coomassie Brilliant Blue G 250 (Serva) in destaining solution (25 % abs. ethanol, 8% glacial acetic acid in Aq. dest.). EIA was carried out after Western blotting onto nitrocellulose filters (NC, Schleicher & Schüll, 0,45 μm) for 40' at room temp. Membranes were blocked with 5% Albumin (Sigma) in PBS, washing steps were done with 0,05% Tween 20 (Merck) in PBS. I. antibody: anti-human α1 acid Glycoprotein (Sigma, 1:200 in 1% Albumin/PBS), II. antibody: anti-goat Immunglobuline conjugated POD (ATAB, 1:1000 in 1% Albumin/PBS) Reaction time was 1 h at room temp. Visualization: 100 μl 4 Chloro-1-Naphthal 2% in Diethyleneglycol; 500 μl saturated O-Toluidine in 7% acetic acid; 500 μl 3% H202 in 40 ml PBS for 10' at room temp.

RESULTS AND DISCUSSION

Pherogram description

Figure 1 shows common (1) and rare (2) ORM-phenotypes after PAGIEF in the pH-range 4.5 - 5.4 detected by immunofixation. For comparison the effect of 5 M urea in the gel is demonstrated.

Without urea homozygotes ORM1 F and ORM1 S show single bands with different IEPs, respectively, whereas hterozygotes ORM1 FS show a double banded pattern. Independently from these phenotype-specific patterns monomorphic gene products appear anodally from the orm1*S band, which seem to be coded for by the locus ORM2.

Unsatisfactory separation of the gene products orm2 and orm1*S however will cause problems with the differentiation of the phenotypes ORM1 F and ORM1 FS.

After separation in gels containing 5 M urea each of the common phenotypes is represented by a characteristic pattern of maior and minor bands. Gene products orm2 appear as faint bands cathodally from the orm1*S band.

Two different rare heterozygous phenotypes were observed, which were designated as ORM1 FS1 and ORM1 SS2. In gels without urea the orm1*S1 product takes a position cathodally from orm1*S. In presence of 5 M urea however the phenotype ORM1 FS1 will be characterized by a band anodally to the maior fraction of orm1*F. Gene products orm1*S2 are clearly separated, independently from the use of urea. In order to characterize variants it seems therefore to be necessary to phenotype ORM also in gels without urea.

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Polymorphism of Orosomucoid (ORM). Formal genetics and population data

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INTRODUCTION

Acid α1-Glycoprotein or Orosomucoid (ORM) represents a plasma sialoglycoprotein with unusually high carbohydrate content. It consists of a single 40 kD polypeptide chain with 181 aminoacids and five heteropolysaccharide side chains (Schmid et al. 1973). Its biological function is described as acute phase reactant (Kushner 1982) with protective responses in inflammatory and infectious states (Friedman 1983).

ORM exhibits a genetically determined polymorphism (Tokita & Schmid 1963) with two common alleles at an autosomal locus (Johnson et al. 1969). The inherited structural variability of ORM can be demonstrated from desialyzed plasma samples (Tokita & Schmid) using PAGIEF (Berger et al. 1980) with addition of urea in the gels (Yuasa et al. 1986), followed by immunofixation (Johnson et al. 1969), lectinofixation (Umetsu et al. 1985) or by enzyme-immuno-assay after Western-blotting.

From the investigation of 159 families with 479 children and of 336 unrelated individuals from southwestern Germany we present data on formal and population genetics of ORM.

MATERIAL AND METHODS

Sample preparation: Serum- or EDTA plasma samples stored up to 6 years at -30°C were treated with Neuraminidase; 3 µl plasma plus 20 µl Neuraminidase (Boehringer, 1 mg/ml Aq.bid.) for 18 hrs at 37°C in a moist chamber.

Polyacrylamide gels (245 x 115 x 0,5 mm) : 2,7 ml Acrylamide (LKB, 28% w/v); 1,2 ml N, N'-Methylenbisacrylamide (LKB, 2% w/v); 0,8 ml Pharmalyte pH 4.5 - 5.4; 10 ml Aq.bid. (alternatively 7 ml Aq. bid. plus 4,68 g urea, Merck); 0,1 ml TMED (Serva, 30 µl/ml Aq. bid.); 0,5 ml Persulfate (Serva, 10 mg/ml Aq.bid.). Polymerization was overnight at room temp. or for 60' at 50°C.

Focusing: Ultraphor with 10°C temp. of cooling water; Anolyte: 0,04 M Glutamic acid; Catholyte: 0,1 M NaOH; Maximum settings: 15 mA, 2000 V; 45' prefocusing with 5W; 6 hrs focusing with 12W; sample application in 3 x 4 mm filter papers (LKB) 2 cm from the cathodal end for 30'.

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The following electrodic solutions were used: 0.1 M Glutamic acid in 0.5 M H_3PO_4 (anolyte) and 0.1 M β-Alanine (catholyte).
Undiluted serum samples were applied at 2 cm from the cathodal end by means of small papers (W 1 MM 7x5 mm).
Focusing was carried out for 270 min (papers were removed after 60 min) with the following maximal conditions: 1500 V, 5W, unlimited mA.
After isoelectric focusing, the gel was stained with the silver staining method of Carracedo et al. (7, 8) using shorter steps.

RESULTS AND DISCUSSION

In table 1 are reported the results obtained with this preliminary study. There is a good agreement between observed and expected values assuming a Hardy-Weinberg equilibrium.

TABLE 1

PHENOTYPE	OBSERVED	EXPECTED
S-S	33	32.0832
F-S	45	46.8289
F-F	18	17.0879
	96	96.0000

ORM^s= 0.5781 ORM^f=0.4219

X^2 =0.1462 .95 p .90 for 1df

In our study the common variants are designated ORM F and ORM S.
The slow banded pattern (we considered the two most cathodally bands of the pattern, with the anode on the top), corresponds to the phenotype ORM S, the more rapidly migrating banded pattern corresponds to ORM F and the double-banded pattern is ORM FS. The corresponding genes are, obviously, designated ORM*S nad ORM*F.
Using the tecnique as described, no difficulties were encountered in the determination of ORM allotypes. The multiple-banded patterns are similar to those described by other Authors.
In comparison to the other electrophoretic techniques, the procedure above described is more simple, cheaper and shorter, for this, we believe that orosomucoid may be routinely used as useful marker both population studies and paternity testing.

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GENETIC STUDY OF SERUM OROSOMUCOID (ORM) POLYMORPHISM BY ULTRATHIN LAYER ISOELECTRIC FOCUSING

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INTRODUCTION

Orosomucoid (ORM), or Alpha-1-acid glycoprotein, serum polymorphism, has not been extensively investigated until now,so that ,it is not routinely used in paternity testing. Genetic polymorphism of human serum orosomucoid can be demonstrated with various electrophoretic techniques such as starch gel electrophoresis after protein purification(1), or immunofixation of neuraminidase treated serum (2), cross immunoelectrophoresis (3), separator isoelectric focusing.

The genetic heterogeneity of serum orosomucoid is due to two codominant alleles at a single autosomal locus: ORM*F and ORM*S which results in the three phenotypes FF, FS and SS (5,6). However, Thymann et al. (4), using separator isoelectric focusing, have demonstrated that the electro= phoretic F band was subdivided in two bands named F1 and F2, so that, five phenotypes were observed.

Recently Carracedo et al. (7,8) have found that the application of silver staining after PAGIF makes the use of immunotechnique unnecessary. Based on these results we have applied the Carracedo's tecnique typing a group of human serum samples. Here we shall outline our procedure, with some modifications respect to Carracedo's technique, and show the results obtained from a preliminary study.

MATERIAL AND METHODS

Sera were obtained from 96 apparently healthy and urelated blood donors of the Blood Bank of Treviso (Veneto, Italy). They were stored frozen in small aliquots at -25°C and tested within some days.

Isoelectric focusing was carried out on polyacrylamide gel (250x125x0.5 mm) on a LKB apparatus connected to a LKB Power Supply, at cool temperature. Each gel was made to a final concentration of acrylamyde solution T=5% and C=3%, sucrose 12% (w/v), carrier ampholytes 3% (w/v) in the 2.5-5.0 pH range. After 15 min of degasation polymerization was achieved with Ammonium persulphate 0.05% (w/v).

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The lectin obtained from the sead weed "fucus tomentosum", reacts with various serum protein including ORM. Other lectin obtained from the beetle "Allomyrina dichotoma", was recently used for the detection of ORM phenotypes (Umetsu et al. 1985). This suggested that some lectins would contribute to the demonstration of polymorphism of some other serum group systems by print lectinofixation after IEF.

The best resolution was achieved using ultranarrow immobilized pH gradients. Furthermore rare additional ORM S subtypes were found, (Fig.3), and although only a few family studies were carried out until now, the codominant inheritance of this allele seems to be clear.

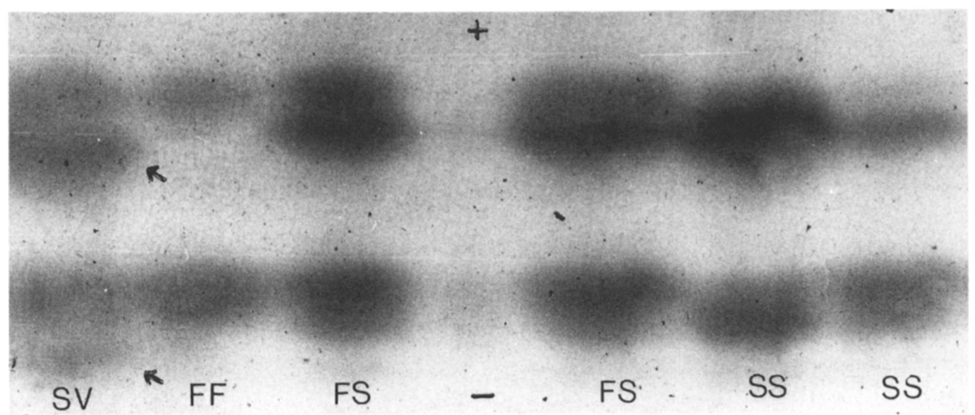

Fig.3 ORM patterns on a shallow immobilized pH gradient (interval of pH 4.2-4.8).

The use of miniaturized gels with the PhastSystem gives also very good results. The adventages of this method are the resolution, reproducibility and the economy. Furthermore the gels can be stored and projected like slides.

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Immunofixation and print lectinofixation were carried out as described above.

RESULTS AND DISCUSSION

Fig. 1 shows the ORM pattern after isoelectric focusing in acidic pH ranges. A clear distinction between phenotypes was found and the silver staining render the use of immunotechniques unnecessary.

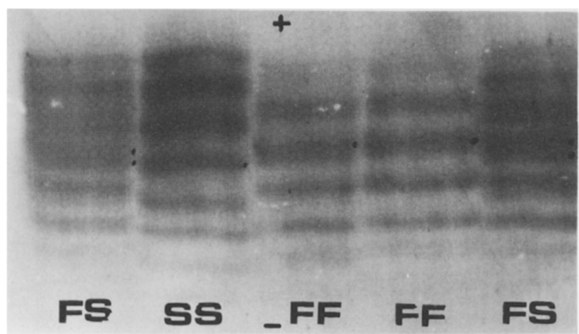

Fig.1 ORM phenotypes by IEF in the pH range 2.5-5 followed by silver staining.

Fig.2 shows the ORM pattern after IEF followed by print immunofixation. The ORM band pattern with the lectinofixation and those with the immuno-fixation are much the same. Phenotypes can be clearly distinguished but a better result is obtained including separators (MES and ACES) as described previously Thymann and Eiberg(1985).

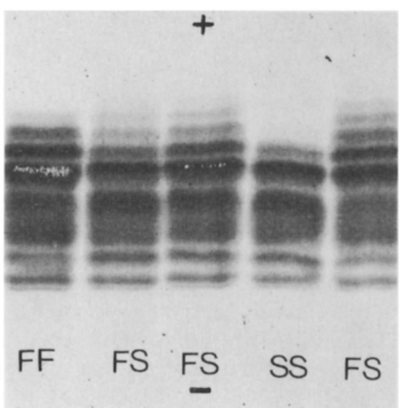

Fig.2 ORM pattern of desialyzed serum after IEF in the pH range 4-6.5 followed by immunofixation

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IEF in acidic pH ranges followed by silver staining

Polyacrylamide gel isoelectric focusing was carried out in 0.4 mm polyacrylamide gels at a gel concentration of T=5% and C=3%. A mixture (1:1) of Pharmalyte 2.5-5 and Ampholine pH 2.5-4 was used in order to obtain an appropriate pH range. Ampholyte concentration was 2.8%. Samples were applied with Whatman 3 filter papers at a distance of 2 cm from the cathode. Isoelectric focusing was carried out at 5 W for 3 h. After focusing the gels were stained with the silver staining method of Carracedo et al. (1983).

IEF of desialyzed serum followed by immunofixation and print lectinofixation

A mixture (1:1) of Ampholine pH 4-6 and Pharmalyte pH 4-6.5 was used. Desialylation of sample was carried out by incubation of 5 µl serum with 20 µl neuraminidase (1U/ml) at 37°C for about 24 h prior to IEF. Samples were applied at a distance of 2 cm from the cathode using Whatman paper. Electric focusing was carried out at 5 W for 3 h.
After focusing immunoprinting was carried out with anti-ORM from Atlantic Antibodies which was diluted 1:1 and applied directly onto the surface of the gel. After 30 min of incubation at 37°C the gels were washed for 24 h with saline and then they were stained with Coomassie R-250. ORM patterns were also obtained by print lectinofixation (time of contact 30 min at 37°C) using a cellulose acetate strip soaked in 0.1mg/mL of a lectin which was prepared by affinity chromatography described previously (Muñoz-Crego 1987). The removed strips were washed for 2h with saline and then stained with Acid Violet 49.
Alternatively miniaturized gels were used, runned with the new PhastSystem (Pharmacia, Uppsala, Sweden). A pH range of 4-6.5 was used. The runs takes 500 Vh or approximately 25 min. Immunofixation was then carried out as described above but the incubation takes only 10 min and the washing step 4 h.

IEF in immobilized pH gradients

Ultrathin layers IPGs were performed as described previously in the LKB application note n.324 (1984). Samples of desialyzed serum were first tested on broad IPG pH 4-5 (buffering/titrating amounts as in the same LKB note). Twofold or more deeper intervals were then derived from such gradient by simple linear interpolation. The pH interval of 4.2-4.8 was particulary useful.

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Improved Diagnosis of Orosomucoid (ORM) Phenotypes by isoelectric focusing in immobilized pH gradients. Comparison with other phenotyping methods

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INTRODUCTION

Alpha-1-acid glycoprotein or orosomucoid (ORM) is a globulin characterized by a very high carbohydrate content (45%), a large number of syalyc residues and an extremely acidic isoelectric point (pI 2.7-3.5)(Schmid 1975).

Although ORM polymorphism was demonstrated twenty years ago (Schmid et al. 1965), it is not habitually used in paternity testing and bloodstain analysis due to the technical difficulties involved in the methods used until recently.

In the last few years, because of the utility of this protein in forensic haemogenetics, ORM polymorphism has been investigated using modern methods. Umetsu et al.(1985), Thymann and Eiberg (1985) and Carracedo et al. (1985) have proposed different methods for ORM analysis, which permit a reliable characterization of ORM phenotypes.

In this paper we present the results of ORM typing by several methods, including isoelectric focusing (IEF) in acidic pH ranges followed by silver staining, print immunofixation, fixation with a lectin from the sea-weed "Fucus tomentosum", IEF followed by immunofixation in miniaturized gels and IEF in immobilized pH gradients.

MATERIALS AND METHODS

Freshly collected blood samples from healthy adults proportionally representative of Galician regional districts were used. Blood samples were collected by syringe into 5 mL tubes containing heparin as anti-coagulant. Plasma was removed and stored at -20°C. Samples were processed within a year after extraction.

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Table 1. Distribution of ORM1 phenotypes and ORM1 alleles in a sample from Southern Germany

Phenotype	Observed n	Observed %	Expected n	Expected %	Allele frequencies
F1	185	38.22	182.82	37.80	ORM1*F1 = 0.6146
F1F2	22	4.55	20.94	4.33	ORM1*F2 = 0.0352
F2	0	0	0.60	0.12	ORM1*S = 0.3492
F1S	202	41.74	207.75	42.96	ORM1*S1 = 0.0010
F2S	12	2.48	11.90	2.46	
S	62	12.81	59.02	12.21	
F1S1	1	0.20	0.60	0.12	
Total	484	100.00	483.63	100.00	

$\sum \chi^{2}$ [a] = 0.4234; df = 3; P > 0.20. [a] Phenotypes with n (exp) below 10 were combined for χ^2 calculation

Table 2. Segregation of ORM1 in 108 families with a total of 112 children

Mating genotypes	Families n	Children n	Genotypes of the children F1	F1F2	F1S	F2S	S	F1S1
F1 x F1	15	16	16	-	-	-	-	-
F1 x F1S	50	53	23	-	30	-	-	-
F1 x F1F2	2	2	2	0	-	-	-	-
F1 x F2S	1	1	-	1	0	-	-	-
F1 x S	2	2	-	-	2	-	-	-
F1S x F1S	14	14	6	-	6	-	2	-
F1S x F1F2	4	4	1	1	1	1	-	-
F1S x S	15	15	-	-	6	-	9	-
F1S x F2S	1	1	-	0	0	0	1	-
F2S x F2S	1	1	-	-	-	1	0	-
F2S x S	1	1	-	-	-	0	1	-
S x S	1	1	-	-	-	-	1	-
F1S x F1S1	1	1	0	-	0	-	-	1
Total	108	112	48	2	45	2	14	1

In 165 cases of disputed paternity, we have thus far observed 16 exclusions in the ORM1 system. This exclusions were confirmed in all cases by further exclusions in other blood group systems. The theoretical chance of exclusion was calculated to be 20.8%. The ORM1 system, therefore, appears to be a useful genetic marker, not only for population studies, but also for paternity testing.

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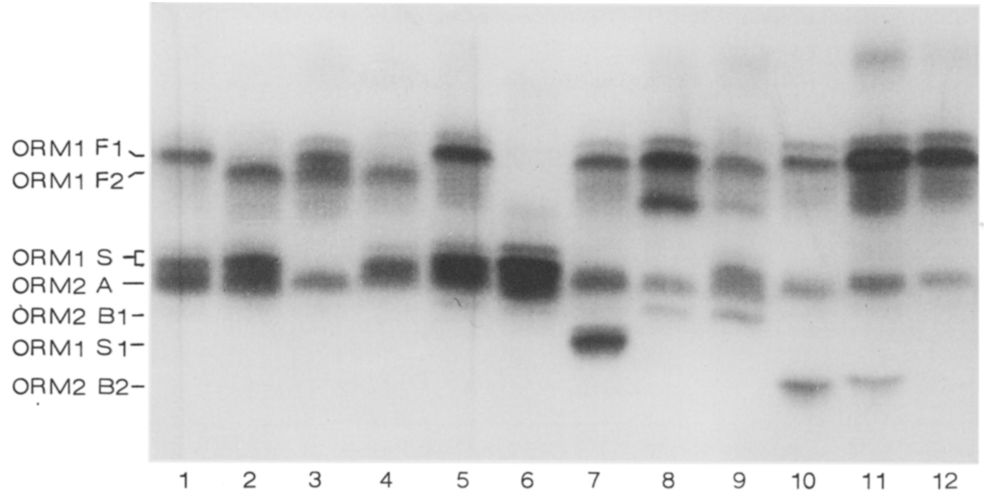

Fig. 1. ORM phenotypes observed by isoelectric focusing on polyacrylamide gel with print immunofixation. Anode is at top. ORM1 phenotypes: lane (1) F1S, (2) F2S, (3) F1F2, (4) F2S, (5) F1S, (6) S, (7) F1S1, (8) F1, (9) F1S, (10) F1, (11) F1 and (12) F1. ORM2 variant phenotypes: lanes (8) and (9) AB1 and lanes (10) and (11) AB2. The other ORM2 banding patterns are monomorphic

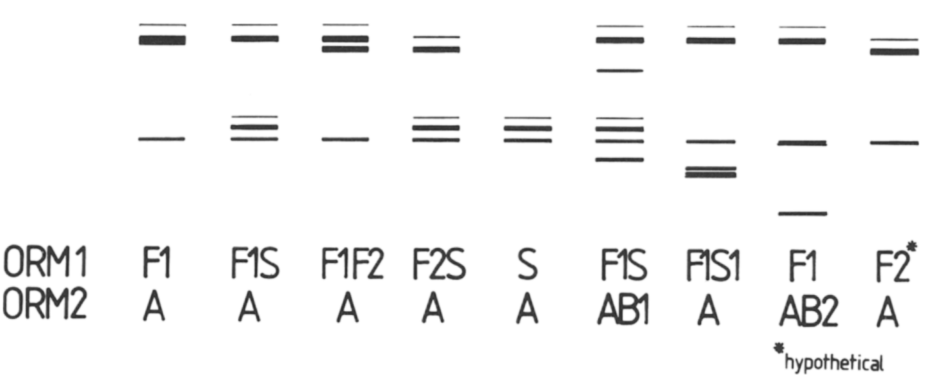

Fig. 2. Diagrammatic representation of the observed ORM1 and ORM2 phenotypes, including the hypothetical ORM1 F2 subtype

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Polymerization was performed within 30 min. after addition of 6 μl TEMED and 180 μl ammonium persulfate (36 mg/ml). 8 μl of serum samples were applied to the gel with Whatman No.3 filter paper pieces at a distance of 2 cm from the cathodal strip. As electrode solutions a mixture of 0.025 M aspartic acid and 0.025 M glutamic acid was used for the anode and 0.1 M NaOH was used for the cathode. Separation was performed at 8 °C for 3 h at a maximum of 2000 V, 16 mA, and 20 W.

Print immunofixation was carried out using a cellulose acetate strip (microsolid, Biotec-Fischer GmbH) soaked with a 1:3 diluted monospecific ORM-antiserum (Behringwerke AG). The strip was placed on the surface of the gel for 150 s. After washing for 20 min. it was stained with Coomassie Brillant Blue R-250.

RESULTS and DISCUSSION

Figure 1 shows the banding patterns of the ORM phenotypes as observed by IEF with subsequent print immunofixation. After neuraminidase treatment of sera the ORM bands focused between pH 4.7 and 4.9. With a narrow pH gradient the common ORM1 F can be differentiated into two subtypes. The most acidic major band is ORM1 F1, the other ORM1 F2. The difference between these two genetic types corresponds to only 0.02 pH units. In the phenotype ORM1 F1F2 a single band is present in the cathodal region which is due to ORM2. In the presence of the allele ORM1*S two major bands can be observed. The anodal band is genetically determined by ORM1*S, the cathodal band belongs to the monomorphic ORM2. In addition three uncommon ORM variants are presented. Two of these have a single band cathodal to ORM2 A. They were classified as ORM2 B1 and ORM2 B2 by family studies. The asymmetrical double band pattern (lane 7) cathodal to ORM2 A is most likely a rare ORM1 variant. This variant phenotype has been tentatively named ORM1 F1S1.

Figure 2 gives a diagrammatic representation of the observed ORM phenotypes, including the hypothetical ORM1 F2 subtype.

Table 1 shows the distribution of ORM1 phenotypes observed in 484 unrelated individuals from Southern Germany. Five common ORM1 subtypes and one variant phenotype were found in this study. The distribution of phenotypes is in good agreement with the Hardy-Weinberg equilibrium. The allele frequencies calculated from the data are: ORM1*F1 = 0.6146, ORM1*F2 = 0.0352, ORM1*S = 0.3492, and ORM1*S1 = 0.001. The population data are very similar to those found in Denmark (Thymann and Eiberg 1986). Asian populations have different frequencies for the ORM1 alleles (Umetsu et al. 1985; Yuasa et al. 1986).

Results from a study of 108 families with a total of 112 children are given in Table 2. In 13 different matings the segregation of genotypes in the children is in accordance with the assumed codominant mode of inheritance.

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Orosomucoid (ORM) Subtyping: Application to Paternity Testing

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INTRODUCTION

An extended genetically determined polymorphism of human alpha-1-acid glycoprotein or orosomucoid (ORM) can be recognized using isoelectric focusing (IEF) in polyacrylamide gel followed by immunofixation or immunoblotting. After neuraminidase treatment of plasma or sera five of the six common ORM phenotypes were observed in population studies indicating the existence of three autosomal codominant alleles (Thymann and Eiberg 1986; Yuasa et al. 1986). According to the nomenclature of Johnson et al.(1969) the three allelic genes were designated ORM*F1, ORM*F2 and ORM*S. The ORM*F gene product can be separated into F1 and F2 only with the high resolving power of IEF. ORM subtyping has been carried out recently in populations from Japan, Denmark and Germany (Umetsu et al. 1985; Yuasa et al. 1986; Thymann and Eiberg 1986; Weidinger et al. 1987). There is evidence for the existence of a second structural locus in the ORM system. ORM has been mapped to chromosome 9 by linkage to ABO, adenylate kinase 1 and delta-aminolevulinate dehydrase (Eiberg et al. 1983).

In this study the ORM polymorphism was investigated in a sample from Southern Germany using a combination of IEF and print immunofixation. The distribution of ORM1 subtypes and alleles is given along with family data. The usefulness of this marker system for paternity testing will be discussed.

MATERIALS and METHODS

The population examined in this study comprised 484 unrelated individuals from Southern Germany. Families were obtained from the paternity testing laboratory. Desialylation of serum samples was carried out prior to analysis by IEF. To 20 µl of serum 10 µl neuraminidase (Clostridium perfringens 1U/mg) were added. Digestion for at least 20 h at 37 ^{o}C was required for essentially complete removal of the sialyl residues.

Isoelectric focusing was carried out with the LKB Multiphor chamber. A 0.5 mm thin polyacrylamide gel (dimension 250x115 mm) consisted of 7.5 ml acrylamide (9.7 g/dl)- and bis-acrylamide (0.3 g/dl) solution, 2 ml glycerol (87%), 0.6 ml Pharmalyte pH 4.2-4.9, 0.4 ml Pharmalyte pH 4.5-5.4, 50 mg sulfonic acid (ACES), and 5 ml destilled water.

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Table 2: Comparison of FXIIIB allele frequencies in several populations

Population (author)	n	B*1	B*2	B*3	B*4	Var
			allele frequencies			
Australia (Board 1980)	245	0.7469	0,0836	0,1693	-	-
Japan						
(Nakamura et al. 1986)	435	0.2977	0.0184	0.6805	-	++
USA						
(Miller et al. 1985)						
Whites	328	0.776	0.088	0.136	-	-
Blacks	178	0.286	0.635	0.079	-	-
Amerindians	88	0.500	0.034	0.466	-	-
Norway						
(Olaisen et al. 1983)	283	0.69	0.15	0.16	-	-
Germany						
NRW (Mauff et al.1983)	334	0.7290	0.1033	0.1632	-	+
Hessen						
(Kühnl et al. 1983)	500	0.73	0.09	0.17	+	+
Bavaria						
(this study 1987)	1496	0.7603	0.0843	0.1534	0.002	-

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Table 1: Distribution of FXIIIB phenotypes and allele frequencies in Southern Germany

Phenotypes	observed		expected		allele frequencies
	n	%	n	%	
FXIIIB 1	861	57.55	864.8	57.81	FXIIIB*1 = 0.7603
2-1	202	13.50	191.8	12.82	
2	9	0.60	10.6	0.71	FXIIIB*2 = 0.0843
3-1	347	23.20	348.9	23.33	
3-2	32	2.14	38.7	2.59	FXIIIB*3 = 0.1534
3	39	2.61	35.2	2.35	
4-1	4	0.27	4.6	0.30	FXIIIB*4 = 0.0020
4-3	2	0.13	0.9	0.06	
Total	1496	100.0	1495.5	99.97	

$Chi^2 = 3.8036$

The value of theoretical exclusion rate for non-fathers is calculated to be 22.7 %.

In table 2 the FXIIIB-allele frequencies in various populations are demonstrated. The allele frequencies of the caucasien populations (Europe, USA, Australia) shows close agreement. In comparison the allele frequencies in Blacks and Mongoloids are extremely different. The frequencies for B*1 are high in Whites and low in Blacks, Asians and American Indians. In Japanese populations (Nakamura et al. 1986) the value of FXIIIB*3 is elevated. The same tendency is observed in Amerindians (Miller et al. 1985). A high FXIIIB*2 frequency is obtained in US- Blacks from Minnesota (Miller et al. 1985).

The limited data available at the present time permit already the conclusion that this system should be very useful for anthropological studies.

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An improved resolution of FXIIIB is obtained by HIEF with IPG and CA. After immunoblotting the bands were obtained by chlornaphtol reaction.

Figure 2 shows the separation with a high degree of resolution. The method of visualisation is very sensitive; the consumption of monospecific antiserum is economical.

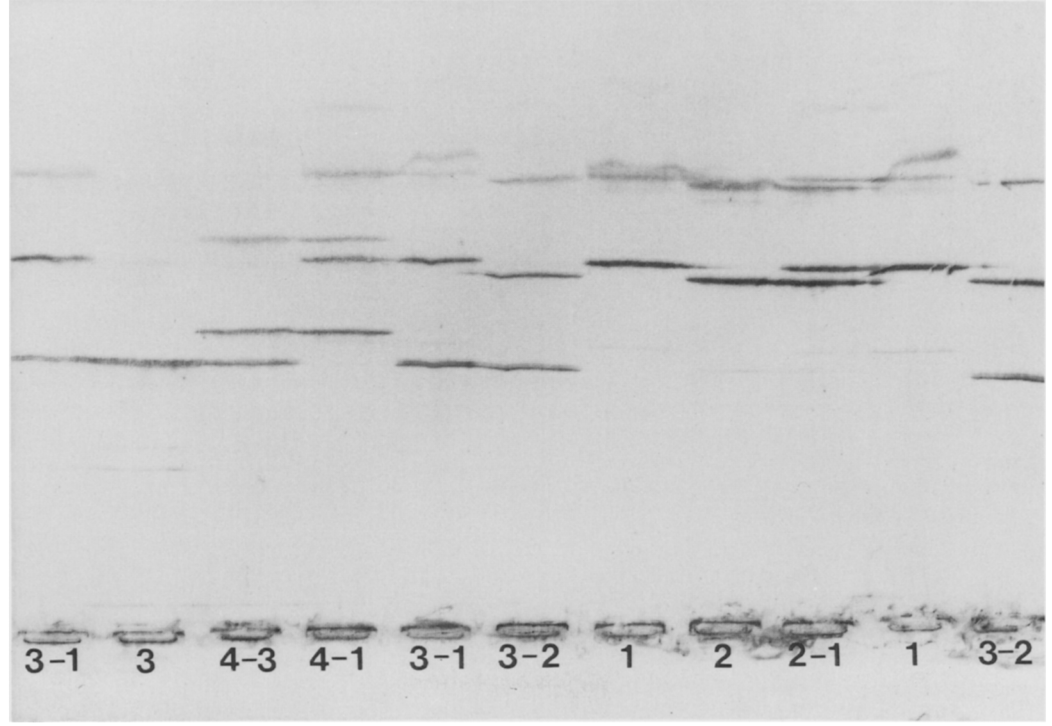

Fig. 2: Hybrid isoelectric focusing with IPG and CA and visualisation by immunoblotting. Anode on top.

Table 1 shows the distribution of FXIIIB-phenotypes and allele frequencies found in a sample of 1496 blood donors of the Blood Transfusion Center of the Bavarian Red Cross. The gene product of FXIIIB*4 was found repeatedly in the combination of B 4-1 and B 4-3. The expected types FXIIIB 4-2 and the homozygous type FXIIIB 4 were not observed yet.

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Blotting

For protein transfer nitrocellulose membrane filter (0.45 µm, Schleicher and Schüll) was placed on the gel surface followed by 5 layers of filterpaper (Whatman N° 1), a glass plate and 2 kg weight for 30 min. Subsequently the NC-membrane was washed three times for 10 min in 5 % skim milk-powder in 0.1 m PBS, pH 7.3.

As first antibody a monospecific FXIIIB-antiserum from rabbit (Behring) was used at a dilution of 1 : 1000, incubation for 5 hrs at room temperature. The subsequent washing procedure was as described above.

As second antibody a peroxidase conjugated anti-rabbit IgG anti-serum (Sigma) was applied. At a dilution of 1 : 2.000 the incubation time at room temperature was 2 hrs, again followed by the washing procedure.

For the development of the bands with chlornaphthol reaction the NC- membrane was soaked in a solution containing 49 ml 0.1 m PBS, pH 7.3; 30 mg 4-chlor-1-naphthol in 10 ml methanol and 1 ml of 0.06 % H_2O_2.

Results and discussion

Figure 1 presents FXIIIB-phenotypes by agarose IEF and immunofixation. The dried Coomassie stained gel was subsequently silver stained. This very sensitive staining procedure is recommanded in particular for FXIIIB typing of older serum or unconcentrated plasma samples.

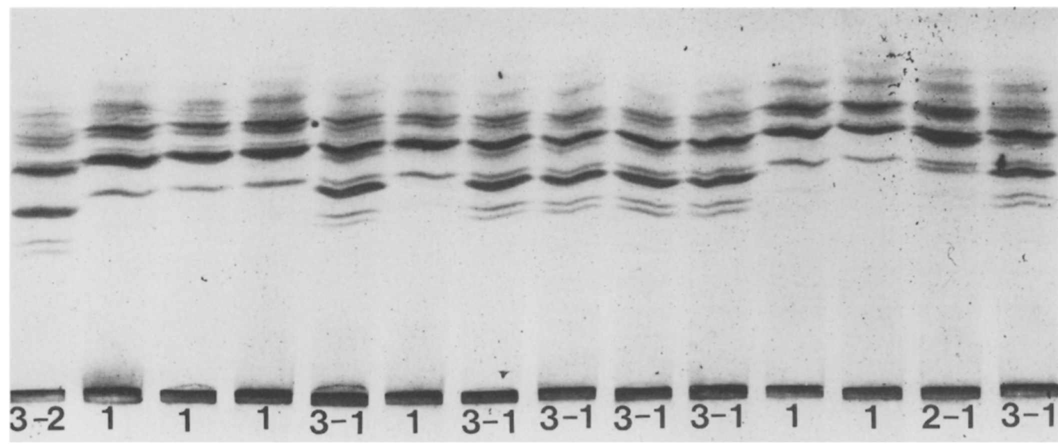

Fig. 1: Immunofixation agarose IEF and visualisation by combined Coomassie and silver stain. Anode on top.

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Improved FXIIIB-Phenotyping by Isoelectric Focusing with Immobilized pH-Gradients

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Introduction

A genetic polymorphism of the B-subunit of the coagulation factor XIII (FXIIIB) was first described by Board in 1980 by agarose gel electrophoresis. By isoelectric focusing on agarose gels we found the four alleles FXIIIB*1, B*2, B*3 and B*4.

As a very sensitive staining procedure we employed the silver nitrate staining method of Willoughby and Lambert (1983). For improved resolution we adapted hybrid isoelectric focusing (HIEF) with an immobilized pH-gradient (IPG) and added carrier ampholytes (CA) (Altland et al. 1986).

Material and methods

Neuraminidase treated serum from 1496 healthy unrelated blood donors from Southern Germany and from 31 families were investigated. All samples were typed first by agarose isoelectric focusing and immunofixation as previously described (Leifheit et al. 1985). Gels were stained with silver nitrate. Questionable phenotypes were examined by IEF with immobilized pH-gradients and subsequent detection by immunoblotting.

Gel preparation

Polyacrylamide gels (T = 5%; C=3%) of 0.5 mm thickness were prepared with an immobilized pH-gradient from pH 5.25 - 6.75 (LKB Appl. Note 324, 1984). After washing and drying the gels were reswelled in destilled water containing 0.5 % carrier ampholytes (pH 5-8).

Isoelectric focusing (IEF)

IEF was carried out for 5 hrs as follows:
1 hr at 600 V; 2 hrs at 5 W; 2 hrs at 25 W. 10 µl samples were applied with a silicon application strip to the cathodal side of the gel. As electrode solutions destilled water was used. The cooling temperature was 10 °C.

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The distribution of the common PLG phenotypes, the PLG variants and the allele frequencies in our population are exhibited in table 2. The allele frequencies of the common alleles are in accordance with the results of other authors (Hobart 1979, Raum et al. 1980, Kühnl und Spielmann 1982, Mauff et al. 1983, Dykes 1983, Weidinger et al. 1985). The variants show a slightly higher frequency. The PLG type A3 was observed fairly often. It is expected that also the homozygote type PLG A3 will be occasionally encountered in our population.

Table 1: Distribution of PLG-phenotypes and allele frequencies in Southern Germany

Phenotypes	observed		expected		allele frequencies
	n	%	n	%	
PLG A	680	50.59	687.1	51.12	PLG*A = 0.7150
AB	541	40.26	527.0	39.21	
B	94	6.99	101.0	7.52	PLG*B = 0.2742
AA.90	1				
AA1	3				
AA3	10				
AM2	4				
AM4	1				
AB1	1				
AB3	1				
		2.61	28.7	2.14	PLG*Var = 0.0108
BA1	1				
BA2	1				
BA3	3				
BM2	1				
BM4	1				
Total	1344	100.0	1343.8	99.99	

$Chi^2 = 0.9342$

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Separation:	cooling temperature: 8 °C 30 min prefocusing, setting 8 W 30 min salt run, setting 0.8 W 75 min setting 1.200 V, 50 mA, 8 W
Immunofixation:	90 min at 37 °C with 1 : 3 diluted PLG-antiserum (Atlantic Antibody)
Pressing:	20 min with filterpaper
Washing:	over night in saline
Drying:	with a heating plate
Staining:	30 min with 0.5 % Coomassie brillant blue solution

Results and discussion

Figure 1 presents the PLG-phenotypes as obtained by IEF on agarose gels followed by immunofixation. The alleles PLG*A and PLG*B determine the three common phenotypes. In addition rare phenotypes are illustrated. The A1, A2 and A3 are arranged in steps. A3 is combined with the common types A and B; A2 and A1 is shown with B. Another variant was observed in the cooridore between A1 and A designated provisionally A.90. In Fig. 1 this variant is demonstrated with both common types (AA.90 is the mother of BA.90). Also the M2- and M4- variants are combined with A and B. The B1-phenotype is slightly displaced to the cathode. The banding pattern of B3 shows a displacement of two bands below the cathodal B-band. All rare phenotypes were confirmed by family investigations.

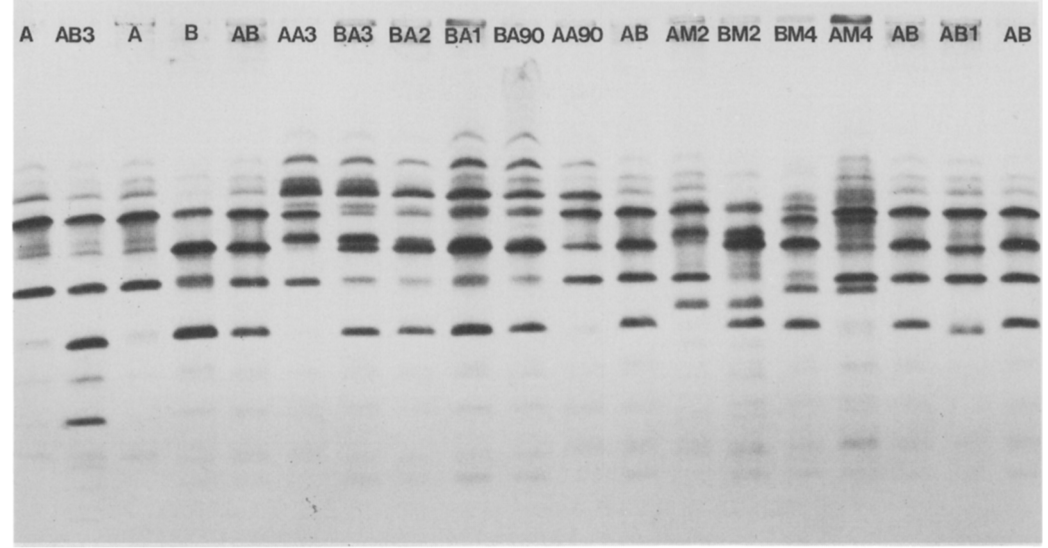

Fig. 1: Isoelectric focusing on agarose gels and subsequent immunofixation of PLG phenotypes. Anode on top.

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Agarose Isoelectric Focusing for Classification of Plasminogen - Variants

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Introduction

Plasminogen (PLG) is the inactive precursor of plasmin. It is a plasmaprotein of the ß-fraction, the quantity in serum varies from 6 - 25 mg/dl. PLG has a molecular weight of 91.000 (Summaria et al. 1976), the part of carbohydrate is 1.5 % (Hayes, Castellino 1979).

The genetic polymorphism has been demonstrated by different electrophoretic methods and immunological or functional detection techniques. It was first described by Hobart (1979) and Raum et al. (1980). The nomenclature followed the proposal of a recent international workshop (Skoda et al. 1986). The common alleles are PLG*A and PLG*B. The variants were classified into three groups: the acidic A- variants, the basic B-variants and the intermediate M-variants.

In this paper the PLG-polymorphism was examined by isoelectric focusing on agarose gels with subsequent immunofixation (Leifheit et al. 1987). We examined a sample of 1344 individuals. We attempted to demonstrate the high resolving power of our classification procedure, in particular, for the separation of the rare variants.

Material and methods

Before electrophoretic separation the sera from 1344 healthy unrelated blood donors from the Blood Transfusion Service of the Bavarian Red Cross were treated with neuraminidase (CPN, Boehringer).

Enzyme treatment:	0.04 U/50 µl serum for 2 hrs at 37 °C
Gel composition:	0.8 % agarose IEF (Pharmacia) 10 % sorbitol dissolved in 18.6 ml dest. water ampholites (LKB) 0.7 ml pH 3.5 - 9.5 0.7 ml pH 5.0 - 8.0
Electrode solution:	anode: 0.25 m acidic acid cathode: 0.25 m NaOH
Application:	8 µl sample on the anodal side of the gel

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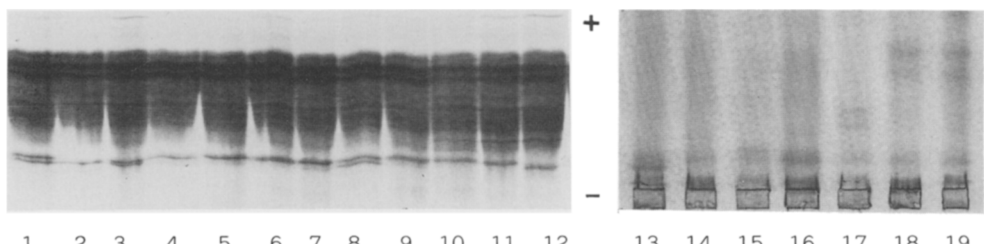

Fig. 1. Tf phenotypes after PAGIF with carrier ampholytes, pH range from 4 to 7 (left) and after IEF with IPG, pH range from 5.20 to 5.75 (right). From left to right: (1) C1-C2, (2) C2, (3) C2-C3, (4) C3, (5) C1, (6) C1-C3, (7) C1, (8) C1-C2, (9) C1-C4, (10) C1-B2, (11) C1-B1-2, (12) C2-B1-2, (13) C3, (14) C1-C15, (15) C1-C4, (16) C1-B4, (17) C1-B3, (18) C1-B2, (19) C1-B1-2[3].

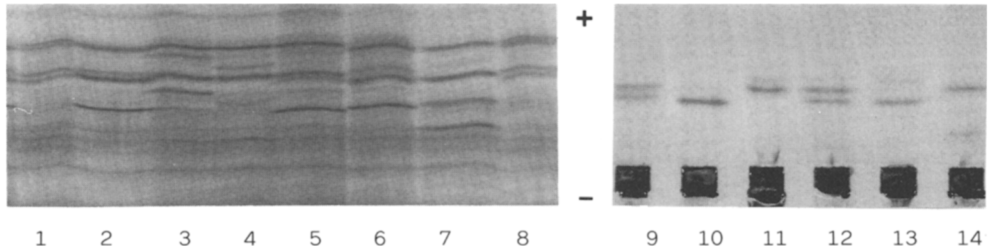

Fig. 2. Pi phenotypes after PAGIF with carrier ampholytes, pH range from 4 to 5 (left) and after IEF with IPG, pH range from 4.45 to 4.75 (right). From left to right: (1) M1M2, (2) M2S, (3) M1N, (4) M1R, (5) M1W, (6) M2S, (7) M3Z, (8) M1M2, (9) M1M3, (10) M2, (11) M1, (12) M1M3, (13) M3, (14) MIN.

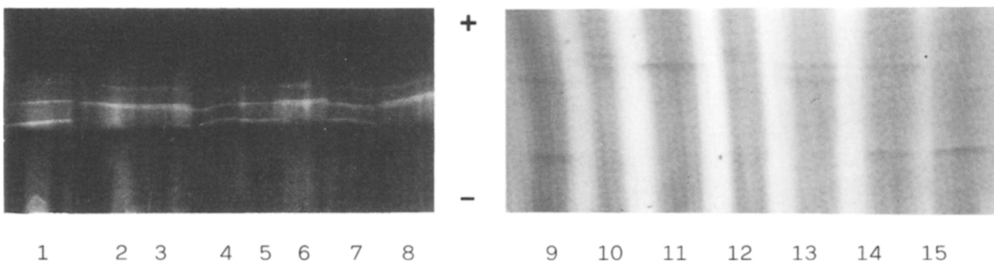

Fig. 3. Gc phenotypes after PAGIF with carrier ampholytes, pH range from 2.5 to 6.5 (left) and after IEF with IPG, pH range from 4.75 to 5.30 (right). From left to right: (1) 2-1F, (2) 1S, (3) 1S-1F, (4) 2-1C2, (5) 2-1S, (6) 1S-1F, (7) 2-1C2, (8) 1S-1C2, (9) 2-1C2, (10) 1S-1F, (11) 1S, (12) 2-1F, (13) 1S-1C2, (14) 2-1S, (15) 2.

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3 Some variants were kindly provided by doctor Giari (Pisa).

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Table 2. Distribution of Pi phenotypes in Ancona

Phenotype	Observed		Expected		Gene frequency
	n	%	n	%	
M1	405	49.82	401.79	49.42	
M1-M2	182	22.39	188.38	23.17	Pi^{M1} = .7030
M1-M3	97	11.93	98.42	12.11	
M2	23	2.83	22.08	2.72	Pi^{M2} = .1648
M2-M3	25	3.07	23.07	2.84	
M3	6	0.74	6.03	0.74	Pi^{M3} = .0861
M1-S	38	4.67	37.95	4.67	
M2-S	12	1.48	8.90	1.09	Pi^{S} = .0332
M3-S	4	0.49	4.65	0.57	
S	0	0.00	0.90	0.11	Pi^{Z} = .0031
M1-Z	3	0.37	3.54	0.44	
M2-Z	1	0.12	0.83	0.10	Pi^{rare} = .0098
M3-Z	1	0.12	0.43	0.05	
M1-rare	13	1.60	11.20	1.38	
M2-rare	2	0.25	2.63	0.32	
M3-rare	1	0.12	1.37	0.17	
Others	0	0.00	0.83	0.10	
Total	813	100.00	813.00	100.00	

$\Sigma\chi^2 = 2.8198$[2] $.80 < P < .90$ 6 d.f.

Table 3. Distribution of Gc phenotypes in Ancona

Phenotype	Observed		Expected		Gene frequency
	n	%	n	%	
1S	270	35.76	268.19	35.52	
1S-1F	130	17.22	126.89	16.81	Gc^{1S} = .5954
1F	14	1.85	15.01	1.99	
2-1S	228	30.20	235.79	31.23	Gc^{1F} = .1411
2-1F	55	7.29	55.78	7.39	
2	56	7.42	51.83	6.86	Gc^{2} = .2622
1S-rare	1	0.13	0.90	0.12	
1F-rare	0	0.00	0.00	0.00	Gc^{rare} = .0013
2-rare	1	0.13	0.40	0.05	
rare	0	0.00	0.00	0.00	
Total	755	100.00	755.00	100.00	

$\Sigma\chi^2 = .8503$[2] $.80 < P < .90$ 3 d.f.

2 In the χ^2 test the variants were pooled with common subtypes.

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proteins: fixation in a mixture of methanol and sulfosalicylic acid, staining with Coomassie Brilliant Blue R-250, destaining in a solution of glacial acetic acid, ethanol and distilled water.

RESULTS AND DISCUSSION

The Figures 1, 2 and 3 show the results obtained and the Tf, Pi and Gc variants classification as we tentatively have made.

It has been possible to carry out the family study of the Gc variants only. The investigation revealed their hereditary transmission.

In conclusion, our work has confirmed the usefulness of IEF with IPG in the resolution of the common subtypes and of the rare variants closely located on them. Moreover it has confirmed the difficulty or impossibility - due to the narrow pH gradient - in the visualization of other types or variants more cathodic or anodic.

Table 1. Distribution of Tf phenotypes in Ancona

Phenotype	Observed n	Observed %	Expected n	Expected %	Gene frequency
C1	420	58.17	419.78	58.14	
C1-C2	216	29.92	213.49	29.57	Tf^{C1} = .7625
C1-C3	37	5.12	40.41	5.60	
C2	25	3.46	27.15	3.76	Tf^{C2} = .1939
C2-C3	12	1.66	10.28	1.42	
C3	2	0.28	0.97	0.13	Tf^{C3} = .0367
C1-rare	8	1.11	7.60	1.05	
C2-rare	2	0.28	1.93	0.27	Tf^{rare} = .0069
C3-rare	0	0.00	0.36	0.05	
rare	0	0.00	0.03	0.01	
Total	722	100.00	722.00	100.00	

$\sum\chi^2 = .9520$[1] $.80 < P < .90$ 3 d.f.

1 In the χ^2 test the variants were pooled with common subtypes.

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Tf, Pi and Gc Variants: a Study by Isoelectric Focusing with Immobilized pH Gradients

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INTRODUCTION

The extremely high resolution of isoelectric focusing (IEF) on immobilized pH gradients (IPG) with ultra-narrow pH ranges has successfully been applied for the analysis of serum proteins polymorphism.

In the former studies (Tagliabracci and Gianni 1986; Tagliabracci et al. 1986; Tagliabracci and Paoli 1987) carried out by polyacrylamide gel isoelectric focusing (PAGIF) with carrier ampholytes we have investigated the transferrin (Tf), alpha-1-antitrypsin (Pi) and group-specific component (Gc) polymorphisms in the population living in Ancona (Middle Italy). Gels were prepared using the specifications advised by Bargagna et al. (1983), with minor modifications. The genic frequencies are shown in Tables 1, 2 and 3.

In order to obtain a better resolution and a sure identification we have utilized the IPG method in the investigation of some variants found in these studies.

MATERIALS AND METHODS

Immobiline pK 3.6, pK 4.6, pK 6.2 and pK 9.3 and N,N,N',N'-tetramethylethylendiamine (TEMED) were from LKB; acrylamide, N,N'-methylene-bis-acrylamide (Bis), ammonium persulfate and Coomassie Brilliant Blue R-250 were from BIO-RAD; glycerol was from MERCK.

The 0.5 mm thin gels were cast with a microgradient mixer (LKB) and in the pH ranges as described by Görg et al. (1983) for Tf, Weidinger and Cleve (1984) for Pi and Cleve et al. (1982) for Gc.

IEF was carried out in a Multiphor II Chamber (LKB 2117) together with a LKB 2197 Power Supply. The running conditions were 10 W, 25 mA and 2500 V overnight at 10°C. The pH gradient in the gel was misured with a Beckman surface electrode immediately afterwards the end of the focusing.

The phenotypes were revealed using the conventional method for serum

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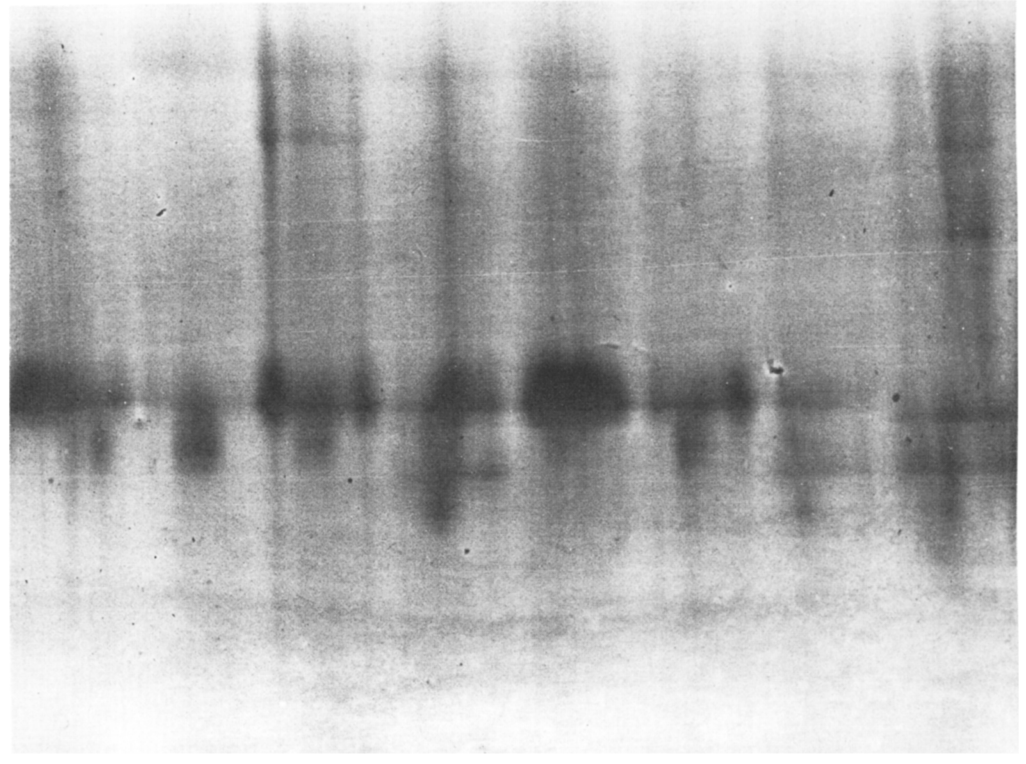

Fig. 1. Transferrin patterns after hybrid IEF on ultrathin layers. From left: C1, C1, C1, C2-1, C1, C1, C2-1, C3-2 (anode is on top).

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voltage 5,000 V). In order not to burn out carrier ampholytes incorporated in the matrix, electrode wicks were employed (10 mM glutamic acid at the anode, NaOH 10 mM at the cathode). Focused gels were fixed in sulfosalycilic acid, then stained with Coomassie. Long stained gels tended to detach from the glass support at the anodal edge, due to the fact that the density medium used for the gradient (either glycerol or sucrose) was being washed away (this does no more occur if more acidic or basic pH intervals are cast). This phenomenon was simply counteracted by adding 5% glycerol to staining and destaining solutions. Stained gels were finally air dried on the same glass supports for permanent record.

CONCLUSIONS

The procedure above detailed allows a fine-quality resolution of transferrin subtypes, exemplified in Fig. 1. Its most outstanding improvements are: a) the molding procedure is fast and performed with wholly reusable materials; b) diluted IPGs in ultrathin-layers are by far less expensive than the traditional slabs; c) the times of separations are noticeably shortened (quite comparable to those required by convective IEF, if a high-voltage power supply is used);d) high-quality performances are obtained with lesser time and costs.
Needless to say, the procedure is largely applicable throughout the scale of pH covered by Immobiline chemicals, as well as for IPGs under denaturing conditions (Pascali et al. 1987).

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polyacrylate- coated (120x 240 x 20mm) plate, with a very thin (0.25 mm) rubber frame between. Acryl-coated plates may be obtained by spraying common glass plates with a polyacrylic paint mixture (e.g. Standocryl 2K, Herberts GmbH, Wuppertal, FRG, diluted 1:4 with MS Verdunnung, Herberts, and supplemented with 50% w/v Standox 2K Harter 66755 Herberts). Acryl-coated plates provide a reusable, heat-resistant, highly-hydrophobic support, which is able to repel the polyacrylamide slab gels. The mold is filled from the top, and the polymerization is allowed to proceed as abitually (50°C for 1 h). After polymerization, when opening the molds, the acryl-coated glasses detach almost spontaneously, while the IPG gels firmly adher to the glass underneath by the interaction between polar immobiline radicals-silica dioxide of glass.

ULTRATHIN IPG SLABS

The acrylate/glass molds virtually allow to pour immobilized gradients in very thin gaskets (0.25 mm thick) since the capillarity generated between the two walls is very low. However, the amounts of dense and light solutions (3,5 ml each, on average) are too small for being evenly mixed by commercially available gradient mixers. For this reason, we used a home-made mixer with small capacity chambers. With this device, we poured a pH interval suitable for transferrin isoelectric point (pH 5.2-5.7, Immobiline amounts drawn by linear interpolation from recipes contained in LKB application note n° 324. Final concentration of immobiline chemicals was half the amount normally advised by the manufacturer (1.5 mEq/pH/L), and the gradient was supplemented with carrier ampholytes of proper range of pH (final CA concentration in gels: 0.5% v/v).

SAMPLE TREATMENT

Neuraminidase (Type VIII, Sigma, 5 U/ml in saline) treatment of serum (7 µl serum + 20 µl neuraminidase) was preferred to ferrous/ferric salts enrichment. Samples (20 µl on average) were soaked on filter paper rectangles and applied cathodally.

ELECTROPHORESIS

The application of ultrathin mixed beds IEF (Gelfi et al 1986), also called 'hybrid' IEF (Altland and Rossman 1985), allows to achieve well resolving separations in half the time normally required for normal-thickness IPGs. Transferrin subtyping was obtained by running gels for 20 KV/hs (initial settings: 200 V, 5 mA, 3.5 W, 10°C, final

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Characterization of transferrin subtypes by hybrid IEF on ultrathin polyacrylamide layers

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INTRODUCTION

Isoelectric focusing (IEF) on immobilized pH gradients (IPGs) is the method of choice for the characterization of a large variety of enzymes and seroproteins (Cleve et al. 1982; Görg et al. 1984a,b; Sutton and Westwood 1984). A vast array of theoretical and practical problems related to the application of IPGs have been by now solved, and well established protocols are available for any needed pH interval of isoelectric separation (Gianazza et al. 1985). However, in spite of the remarkable refinements of IPGs technology, a number of problems still hinder immobilized gradients to be used as a true routine procedure of electrophoresis. Noteworthy, the IPGs gel casting procedure is as much time-expensive as to discourage its universal use. IPGs are currently performed on polyacrylamide gels as thick as 0.5 mm, whose casting requires to assembly a repel-silanised glass plate with either a bind-silanised glass or a GelBond film, and a rubber spacer between. Silanisation happens to be one of the most tedious and time-wasting steps and, every now and then, even carefully silanised glasses do not prevent gels to tear out when opening the molds.
Another drawback is in the very long separation times required for ultranarrow pH intervals (< 1 pH unit).
For all these reasons, each IPGs experiment usually takes almost two days to completion.
Finally, immobiline chemicals are more expensive than commercial convective pH intervals.
We recently sought to circumvent these drawbacks, by resorting to an extremely simplified gel casting procedure, enabling to perform diluted (1.5 meqv/pH/L) IPGs on ultrathin slab gels, with considerable times and costs saving. The procedure is here described for the characterization of transferrin subtypes.

METHACRYLATE/GLASS MOLDS

The molding procedure is based on the use of a common (unsilanised) glass plate, assembled together with a

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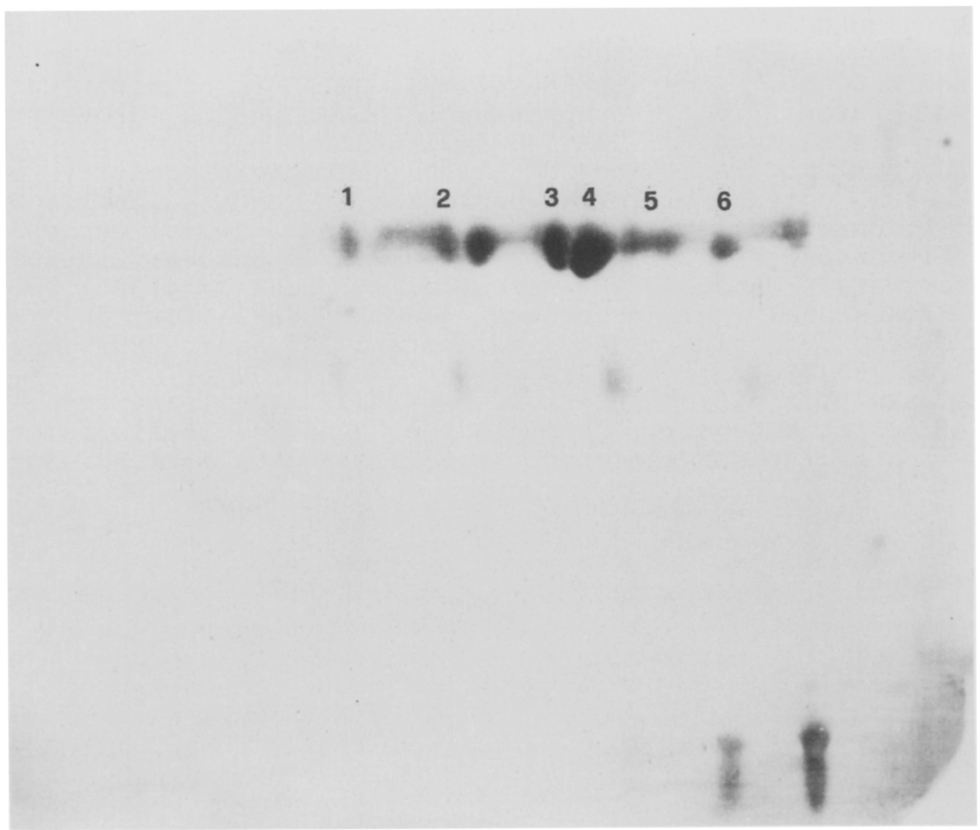

Fig.2. Two-dimensional pattern of antitrypsin spots by a high-resolution 2-D PAGE protocol (IEF pH range 4.2-4.9; SDS PAGE spanning over 12.3-13.5 % T). M2M3 subtype is shown.

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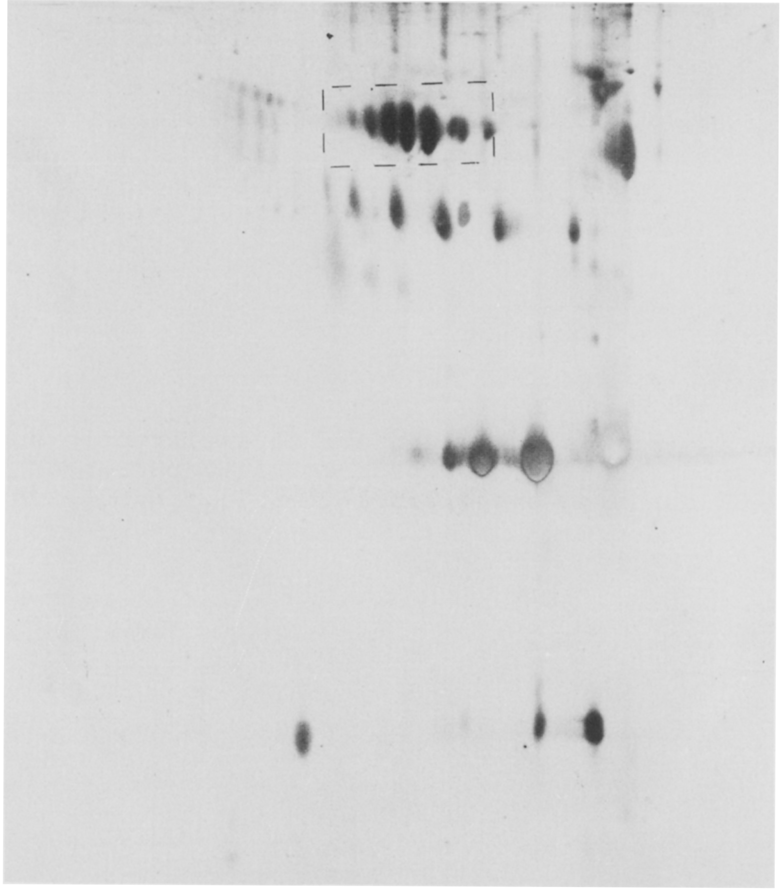

Fig. 1. Two-dimensional pattern of acidic seroproteins (IEF in the range of pH 4-6, SDS PAGE 10-20% T; anode is on the left, higher Mr components uppermost; PI train of spots is indicated). Heterozygous MS is easily recognized.

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In a second set of experiments, ultranarrow-range carrier ampholytes (pH 4.2-4.9) were used, spanning just around the isoelectric points of the PI isoforms in the first dimension.
The application of this high-resolution 2-D PAGE protocol (ultranarrow IEF gradient and narrow T% SDS PAGE) resulted in some improvements of the patterns of Alphal antitrypsin, evidently due to the use of ultranarrow pH gradients in the first dimension. Distances between the spots were enlarged and some minor components of Antitrypsin microheterogeneity were revealed. Conversely, there were only minor advantages by the use of narrow polyacrylamide gradient gels in the second dimension.
As shown in Fig. 2, additional components of microheterogeneity became apparent in the heterozygous patterns, along the pH axis. Conversely, no further components of microheterogeneity appeared along the Mr axis, complying with the hypothesis that M variants are devoid of mass variability.
The meaning of these differences is difficult to evaluate. Uncertainties arise from the fact that each spot may not be granted as the product of a single M allele, presumably because of some overlapping of different allele products during the SDS PAGE dimension.
On the other hand, by assuming each pattern as a whole, a diagnosis of subtype is possible.

CONCLUSIONS

From these experiments we conclude that:
a) Unlike PIM, PIS and PIZ, M subtypes are devoid of significant mass hererogeneity (i.e. they possess almost the same rate of carbohydrate moyety).
b) Differences between M subtypes may be assumed to be only due to charge variability.
c) Comparisons between 2-D PAGE patterns with convective isoelectric dimension (CA IEF) are somewhat unadequate to prevent different spots to merge one into another during the second dimension. This considerably impair the resolution of the technique.
d)The technique of 2-D PAGE has been recently proposed for the simultaneous typing of several genetic markers in father/mother/child trios (Asakawa et al. 1985). However, owing to the substantial loss of informativeness in each system, its adoption in the routine of paternity testings is not realistic. As shown by the case of PI system, a share of charge variability is lost during the second dimension. Improvements in 2-D PAGE protocols are still needed. They will hopefully derive from the application of immobilized pH gradients in the IEF dimension.

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ampholytes and 9M urea) adhered to GelBond plastic film. Gradients of nominal pH 4-6 and 4.2-4.9 were in turn used, with a voltage of 7000 V/hs (final voltage 1500 V/1h). After fixing (10% W/V sulfosalicylic acid), staining (0.5% Coomassie R250) and destaining (10:10:80 ethanol:acetic acid:water) gels were dehydrated, cut in strips and frozen. Prior to the second dimension, strips were equilibrated for 10 min. in 0.25% M Tris/Hcl buffer pH 6.8 containing 2.1% SDS and 10% glycerol, then embedded onto the second dimension gel's edge and sealed with 1% W/V agarose. Second dimension gradient gels gave best results with a 12.5-13.5% T. Electrophoresis (25 mA/gel, maximum voltage 500 V) was continued until 45 min. after the Coomassie dye front had left the gels (about 5 h for 10-20% gradients, 3 h for the narrower's); proteins were then visualized using a silver stain procedure.

RESULTS

We identified PI components by a comparison of immunoprecipitates patterns with 2-D PAGE images of native serum: five major anodic spots were identified, and two minor on their cathodic side, with an apparent Mr slightly decreasing towards the cathode. A range of pH 4-6 was employed to separate M, S and Z subtypes; it became apparent that intensities of spots would progressively fade from M to S to Z, with slightly changed distances. Z patterns possess especially strong spots n° 4 and 5, while S patterns have selectively intense spots n° 3, 4, 5; heterozygous MS duplicate spots n° 6 and 7, whereas other components partially merged into adjacent M spots. Since the three allele produce one-to-one corresponding trains of spots, the discrimination of MS an MZ heterozygous is quite simple. This procedure did not show any mass variability between M1, M2 and M3 allele product, whose trains of spots were yet different along the pH axis. Finally, the application of an ultranarrow gradient of pH along the first dimension resulted in an improved 2-D separation of M subtypes (see discussion).

DISCUSSION

Fig. 1 and Fig. 2 illustrate two different stages of 2-D PAGE experiments.
Using carrier ampholytes in the range of pH 4-6, M, S and Z phenotypes are readily identified (Fig. 1). However no considerable differences could be detected between PIM subtypes.

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Classification of alphal antitrypsin phenotypes by high-resolution two-dimensional electrophoresis (2-D PAGE)

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INTRODUCTION

The ability of two-dimensional gel electrophoresis (2-D PAGE) to visualize soluble proteins is well documented (Anderson and Anderson 1977). By this tecnique, exploiting charge/mass differences to separate polypeptides, many polymorphic serum proteins were exactly located in 2-D PAGE maps of human serum (Anderson and Anderson 1979). The main advantages of this procedure are: first, a better molecular characterization of mutants is obtainable, secondly a simultaneous detection of several proteins is possible in the same slab gel;finally, the monitoring of non-conventional phenotypes,as the null variants (which would otherwise be difficult to detect) could be obtained (Asakawa et al. 1985).
Alphal antitrypsin (PI) has a high electrophoretic variability, due to aminoacidic substitutions along the proteic backbone and to three different carbohydrate side chains as prosthetic groups (Vaughan et al. 1982).
In this paper we sought to improve the detection of PI molecular variants by a 2-D PAGE procedure. M, S and Z electrophoretic types were easily discriminated. A modified procedure, based on the use of ultranarrow pH gradients in the IEF dimension together with SDS PAGE electrophoresis suitable for the Mr of PI, was finally employed, with a view to separate M subtypes.

MATERIALS AND METHODS

Sera were obtained from healthy donors and typed for PI. Prior to the first dimension step, 9 M urea and 0.5 to 1% 2-mercaptoethanol were added to the samples. Immunoprecipitates were obtained from sera of self-assessed homozygous products additioned with specific antiserum to PI, by centrifugating after each addition and finally discarding the supernatant. Immunoprecipitate pellets were solubilized in 9M urea/2-ME immediately prior to electrophoresis. Isoelectric focusing ran on ultrathin (0.3 mm thick) polyacrylamide (T=5%) gels (containing 5%

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of Hp 1 molecules. This is reflected in the Hp 1 band of the 2-1 type. Hp 2 components, on the other hand, are fully hybridised in all cases, so that free Hp 2 molecules do not exist in the mixed type. However, ring closure of $\alpha_2\beta$ units resulting in the Hp 2 molecule cannot be fully avoided in the case of imbalance of gene products at the expense of Hp 1. Hence, Hp 2 molecules will have to be expected in addition to the hybrid molecules of the 2-1 type. An attempt was made by means of high-resolution polyacrylamide gradient gel electrophoresis to make comparisons between the Carlberg type and an artificial Hp 2/2-1 mixture, since the corresponding Hp 2-1 and Hp 2 polymers, such as $(\alpha_2\beta)_2$. $(\alpha_1\beta)_2$ or $(\alpha_2\beta)_4$, each exhibited a molecular weight difference of some 13,000 Dalton. The separation images thus obtained were absolutely identical, which confirmed the conceptual model. Amino acid analysis of the gene products

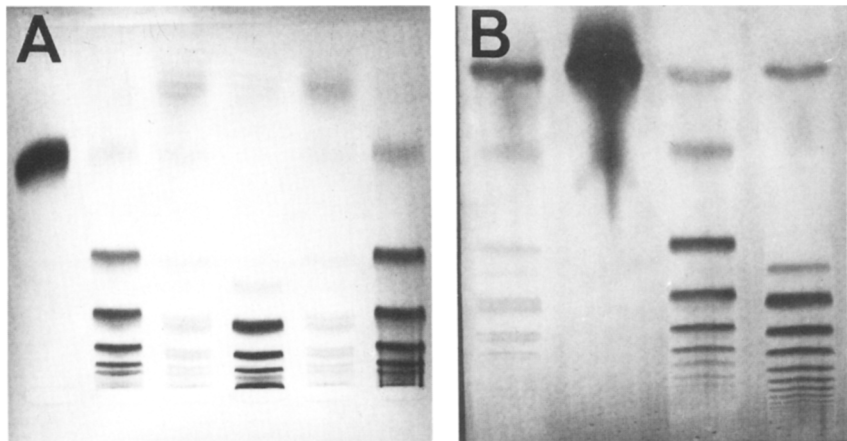

Fig. 3. Representation of Hp types in polyacrylamide gel electrophoresis (A) and polyacrylamide gradient gel electrophoresis (B). Types from left to right are A: Hp 1, 2-1, 2-1Ca, 2, 2-1Ca and B: Hp2/2-1 mixture, artifact, 2-1, 2

probably will be necessary to definitely clear up, if the Carlberg variant is an expression of point mutation with an amino acid sequence different from the α_1S-peptide or an expression of a mutated regulator gene (no segregation so far being observed).

Interesting enough, there are alleles also for the Hp 2 gene product which encode a α_2-peptide with reduced concentration: modified Hp 2 genes (Hp*2M). These genes possibly are recombination products of Hp*Ca genes with Hp*2FS genes. From the polymerisation diagram (Fig. 2) one can easily predict the Hp 2M-1 type which is characterised by a shortened polymer series.

So, the following concept should apply to the specific case of paternity assessment: No definite ruling can be made as to whether the child is homozygous Hp 1S or heterozygous Hp 1Ca-1S. The defendant's paternity, consequently, cannot be excluded in the Hp system.

The authors of this paper, on the basis of their own results, should like propose that the Hp Ca variant in future should be renamed more informatively Hp 2-1Ca.

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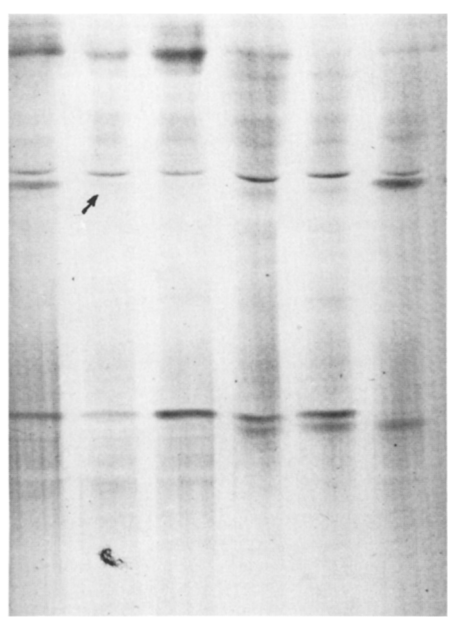

Fig. 1. Representation of Hp subtypes; types from left to right: Hp1S-2FS, 1Ca-2FS, 2FS, 1F-2FS, 1F-2FS, 1F-1S. The arrow points at the 1Ca band

Now, if one looks at the structure of haptoglobin polymers, the reduced offer of the Hp 1 component must have considerable consequeces for hybridisation of the Hp 2-1 molecule (Fig. 2).

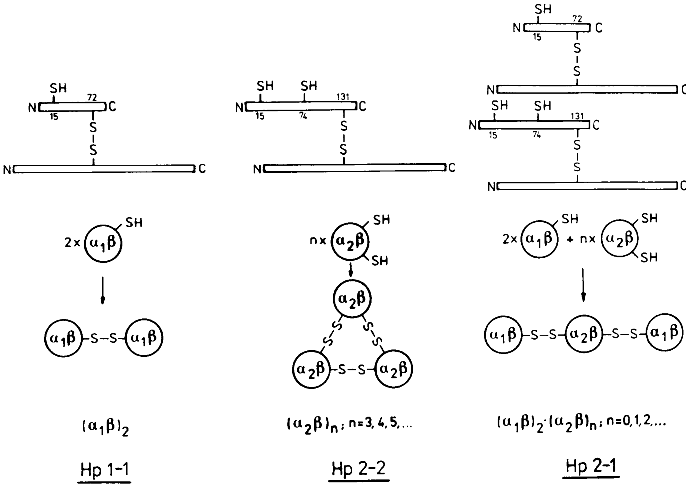

Fig. 2. Diagram of structure of haptoglobin polymers (modified after Bowman and Kurosky)

The primary gene products of Hp*1 and Hp*2 on a heterozygous basis are normally formed in identical quantity, which for higher 2-1 polymers results in a surplus

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Studies into Structure and Inheritance of the Genetics Hp Ca-Variant

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Several theories exist on the nature of the Carlberg variant (Galatius-Jensen, 1958): Parker and Bearn (1963) assumed that possibly there was a gene complex consisting of a normal Hp*1-, a normal Hp*2- genes. According to studies by Giblett (1964), Hp Ca was characterised by reduced production of Hp 1-units. Henningsen and co-workers (1977) confirmed Giblett's assumption and produced evidence to the effect that reduced production applied exclusively to the gene product of the Hp*1S-gene. That phenomenon has been attributed to a mutated regulator gene that is closely linked to the Hp*1S-gene.

We received for paternity testing sera of individuals involved which were to be subtyped in the Hp system. The phenotype situation was favourable, according to the outcome of preparatory tests (Table 1). Diagnosis of the rare Carlberg variant obviously failed for too low haptoglobin concentrations in the serum of the defendants, and the Carlberg variant, after all, is characterised by a complex and usually poor band pattern. A 2-1S-like pherogram, conspicuous for absence of the heterosis effect, was recorded from subtyping (Patzelt and Schröder, 1985). A "normal" α_2FS band was paralleled by a very poor α_1S line which, however, could not be overlooked (Fig. 1). For type identification we used high-resolution, concentrating polyacrylamide gradient gel electrophoresis, and we obtained a separation image close to the Carlberg variant, as repeatedly described in the literature (Prokop and Geserick, 1986).

Table 1.

	Starch gel electrophoresis	Isoelectric focusing	Gradient gel electrophoresis
Mother	Hp1-1	1 S	1-1
Child	Hp1-1	1 S	1-1
Defendant	Hp2-1?	(1 S)-2 FS	Ca

The views of Giblett (1964) as well as of Henningsen and co-workers (1977) on the deficiency of the Hp 1S gene product of the Carlberg type have thus been confirmed by our own results which had been obtained from isoelectric focusing. Yet, we consider probable the existence of a gene Hp*1Ca, its product having an isoelectric spot almost or fully identical with that of α_1S-peptide. Such gene products result from point mutations which are accompanied by exchange of similar amino acids.

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Table 2. Segregation of Hp subtypes in 27 families with 62 children

Matings	n	Children (n=62)						
		1F	1S-1F	2FS	2FS-1S	2FS-1F	2FS-2SS	2SS-1S
1F x 1F	1	3	-	-	-	-	-	-
2FS x 2FS	4	-	-	13	-	-	-	-
2FS x 2FS-1S	3	-	-	3	2	-	-	-
2FS x 2FS-1F	5	-	-	8	-	3	-	-
2FS x 2SS-1F	2	-	-	-	-	3	0	-
2FS x 1F	1	-	-	-	-	2	-	-
2FS x 1S	2	-	-	-	4	-	-	-
2FS-1S x 2FS-1F	3	-	3	1	3	1	-	-
2FS-1S x 2SS	1	-	-	-	-	-	1	1
2FS-1S x 2FS-2SS	1	-	-	0	5	-	1	0
2FS-1F x 2FS-1F	1	1	-	0	-	0	-	-
2FS-1F x 1S-1F	1	0	-	-	1	0	-	-
2FS-1F x 1S	1	-	2	-	0	-	-	-
2FS-2SS x 2FS-1F	1	-	-	0	-	1	0	-
Total	27	4	5	25	15	10	2	1

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Table 1. Distribution of Hp phenotypes in Sweden

Hp-types	Observed n	Expected n	Gene frequency
1S	37	30.20	$Hp^{1S} = 0.231$
1F	9	13.73	
1F-1S	44	40.72	$Hp^{1F} = 0.156$
2FS	192	183.82	
2FS-2SS	25	26.27	$Hp^{2FS} = 0.571$
2FS-2FF	0*	0.58	
2SS-2FF	0*	0.05	$Hp^{2SS} = 0.041$
2SS	0*	0.94	
2FF	0*	0.00	$Hp^{2FF} = 0.001$
2FS-1S	131	149.02	
2FS-1F	104	100.46	
2SS-1S	11	10.65	
2SS-1F	10*	7.18	
2FF-1S	1*	0.23	
2FF-1F	0*	0.16	
Total	564	564.01	

$\chi^2 = 6.5$ $0.1<p<0.2$ 4 d.f.

* Subtypes are pooled in one group according to the table for the χ^2-analysis.

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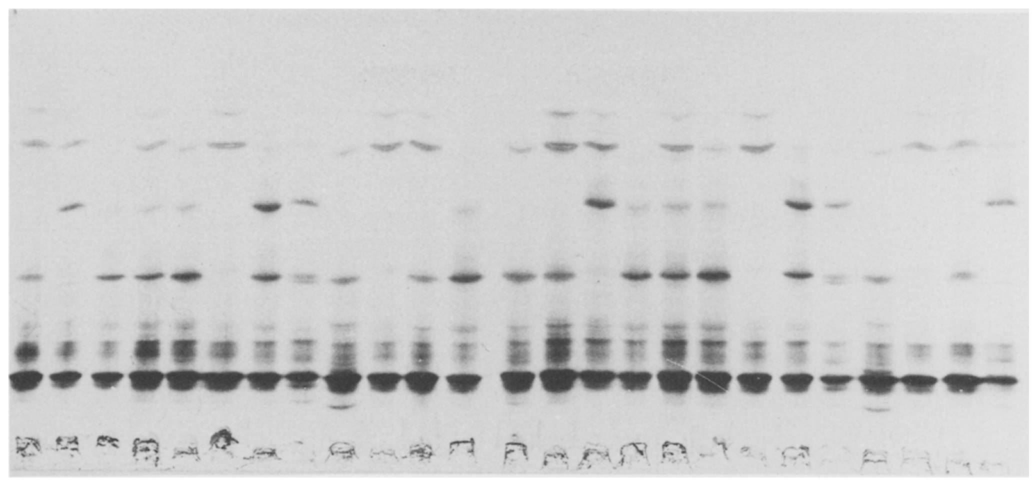

Fig. 1. Haptoglobin phenotypes in neuraminidase treated serum samples revealed by isoelectric focusing and immunoblotting procedures. Hp phenotypes from left to right: 1F-1S, 2SS-1S, 2FS, 2FS-1S, 2FS-1F, 1S, 2FS-2SS, 2SS-1F, 2FF-1F, 1S, 1F-1S, 2FS, 1F, 1F-1S, 2SS-1S, 2FS, 2FS-1S, 2FS-1F, 1S, 2FS-2SS, 2SS-1F, 2FF-1F, 1S, 1F-1S and 2SS.

The Hp allele frequencies agree well with the data published from other populations in Europe (Thymann 1977; Shibata et al. 1982; Teige et al. 1985). Hp subtyping of 113 mother-child pairs has been investigated. Unexpected combinations in the material are not found. The data are in full accordance with the postulated genetic model of an autosomal locus with codominant alleles.

The results of the examination of 27 families with 62 children are given in Table 2. In each combination group of parental types, the segregation of phenotypes in the children is in accordance with the assumption of autosomal, codominant inheritance.

The isofocusing/immunoblotting method suits a routine laboratory very well because no purification of the haptoglobin molecule prior to isofocusing and only one dimensional electrophoresis are required. The determination of Hp subtypes extends the number of common alleles from two to five and the theoretical chance of exclusion of non-fathers from approximately 18 % to 30 %.

The Hp system is now very informative and classification results are highly reproducible. The system is useful for both population studies and cases of disputed paternity.

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Sample Treatment

Serum samples were reduced and neuraminidase treated according to Teige et al. (1985).

Gel Preparation

110 x 240 x 0.5 mm polyacrylamide gels (T=4 %, C=3 %) were cast on Gel Bond (FMC Corporation, USA) film and polymerized with TEMED and ammoniumpersulphate. The gels contained 3.4 % of an Ampholine (LKB, Bromma, Sweden) mixture of 3 parts pH 3.5-10, 2 parts pH 4-6 and 1.5 parts pH 6-8.

Isoelectric Focusing

The anode solution was 1 M H_3PO_4 and the cathode solution was 1 M NaOH. The cooling temperature was +10°C. The gels were pre-focused for 1 h with maximum settings at 500 V and 8 W before the samples were applied (Wh1 7x4 mm) 0.5 cm from the cathode. The filter papers were removed after 45 minutes focusing with maximum settings at 1300 V and 10 W. Then the isofocusing was continued for 1.5 h with maximum settings at 1800 V and 16 W.

Blotting Procedure

The proteins were transferred to nitrocellulose sheets (Bio-Rad, Richmond, USA) by passive blot for 1 h at room temperature. Thus the nitrocellulose paper was presoaked in 40 mM Tris, 192 mM glycine, 0.01 % SDS buffer, pH 8.7 before placing on top of the gel, followed by layers of filter papers wetted in the blotting buffer, and some layers of dry filter papers.

Visualization

The nitrocellulose paper was washed in 0.15 % Tween 20, 48 mM Na_2HPO_4, 18 mM KH_2PO_4, 147 mM NaCl buffer, pH 7.2 (PBS-Tween buffer) for 2 h. The paper was shaken over night in a solution of peroxidase conjugated goat-anti-human Hp (Cappel, Worthington) diluted 6/1000 with PBS-Tween buffer and washed for 1 h in PBS-Tween buffer. The development was performed in 50 ml of a 0.2 M NaCl, 59 mM Tris-HCl buffer, ph 7.4, 30 mg 4-chloro-1-naphtol, 2 ml acetone and 30 μl 20 % H_2O_2. The colour reaction was stopped with water.

In contrast to the method by Teige et al. (1985) one antibody is used instead of the two-step antibody technique whereby the method will be less time-consuming and laborious.

RESULTS and DISCUSSION

The phenotype pattern is shown in Fig. 1. The distribution of Hp phenotypes and gene frequencies in the population in Sweden are presented in Table 1. The population was in Hardy-Weinberg equilibrium with a chi-square = 6.5 $0.1<p<0.2$ at 4 d.f.

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The Distribution of Hp Subtypes in Sweden

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INTRODUCTION

Smithies et al. (1962) showed the existence of haptoglobin polymorphism. Population studies of the polymorphism have in general been restricted to the identification of the three phenotypes Hp1, Hp2-1 and Hp2. Technical improvements have made it possible to detect Hp subtypes.

The haptoglobin molecule consists of α- and β-chains linked with disulfide bridges. The isoelectric heterogeneity of the β-chain is due to its content of sialic acid. The α-chain is polymorphic, and the most common suballeles 1S, 1F, 2FS, 2SS and 2FF may be separated by isoelectric focusing.

In 1985 Teige et al. presented a method for Hp subtyping, which is very well adapted for routine investigations of cases of disputed paternity.

The aim of this study is to present the distribution of haptoglobin subtypes and allele frequencies from unrelated adult Swedes. A family and a mother-child material are also presented.

MATERIAL and METHODS

Serum samples from 564 unrelated Swedish adults and a mother-child material consisting of 113 pairs were investigated. All persons were involved in paternity cases. Sera from 27 families with 62 children were also examined. The family material originated from northern Sweden and was involved in a diabetes mellitus (IDDM) investigation, including IDDM families as well as control families.

After arrival at the laboratory the serum was separated from the blood cells by centrifugation and stored at +4°C. The subtypes were determined twice or more.

The determination of Hp subtypes was carried out by isoelectric focusing followed by enzyme linked immunoblotting technique mainly according to Teige et al (1985).

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tion of population genetic data and variants. Ärztl Lab 33:85-87

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(6) Hp 1F-1S.

First Johnson subtype pattern (1) shows beside hp 1S a second intense band cathodal to the 2FS-position and an additional faint band in the 2FS-position. The second Johnson subtype pattern (4) is composed of a clearly visible hp 1F- and two further bands both anodal to the 2FS-position. With respect to staining intensity the more anodal protein seems to represent the main band.

To conclude, Hp subtyping can be done routinely by ion exchange chromatography, reductive cleavage and separation by IEF. Hitherto unknown α-chain variants could be detected by this method. Triplication of α-chains in Johnson phenotypes can be simply verified by SDS-acrylamide electrophoresis. Two different subtypes of Hp Johnson were identified by IEF.

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Fig. 5.SDS electrophoresis of isolated Hp. (1) Hp J-1, (2) Hp 1, (3) Hp 2 and (4) Hp 2-1

0.5 mm thick acrylamide gels were poured with a linear gradient T5%-T25% at constant C=3%. 0.375 mol Tris-HCl pH 8.8 with 0.1% SDS was used as gel buffer. The bridge buffer consisted of 0.025 mml Tris, 0.05 mol glycine and 0.1% SDS. Gels were run at 5°C with maximum settings of 1400V, 50mA and 40W. Hp was isolated from 200ul serum as described and treated with 100ul 1% SDS and 0.05% DTT in 10 mmol Tris-HCl pH 8.8. Figure 5 shows a Hp J-1 together with the main phenotypes Hp 1 (2), 2 (3) and 2-1 (4). From these four persons two different subtype patterns were obtained when reduced and alkylated Hp was subjected to IEF (Fig. 6)

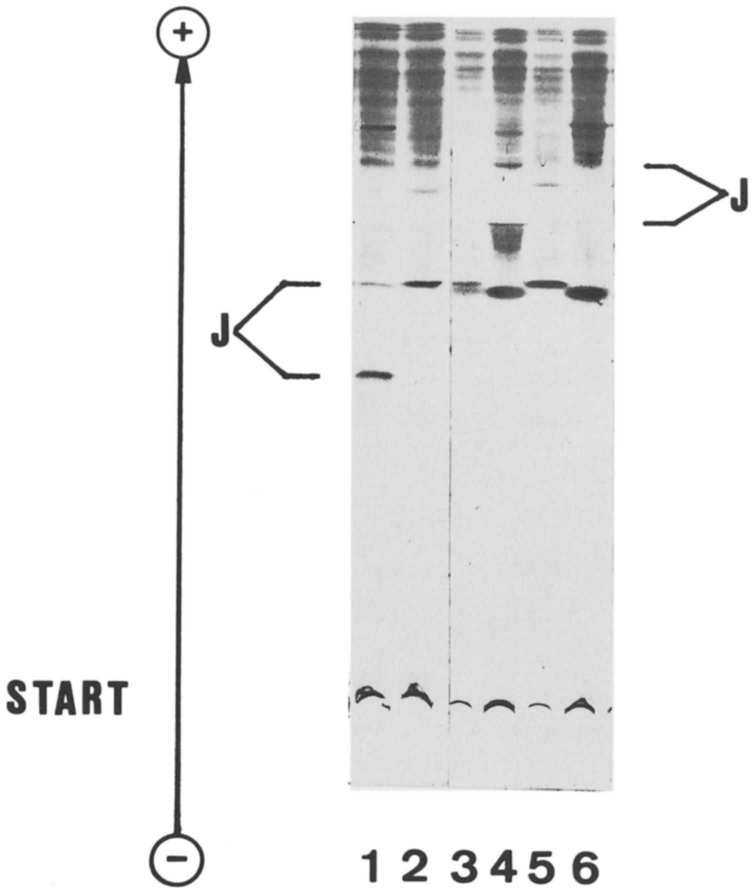

Fig.6.Two different Johnson subtypes as revealed by IEF. (1) Hp J-1S, (2) Hp 2FS-2FS, (3) Hp 2FS-1F, (4) Hp J-1F, (5) Hp 2FS-2FS,

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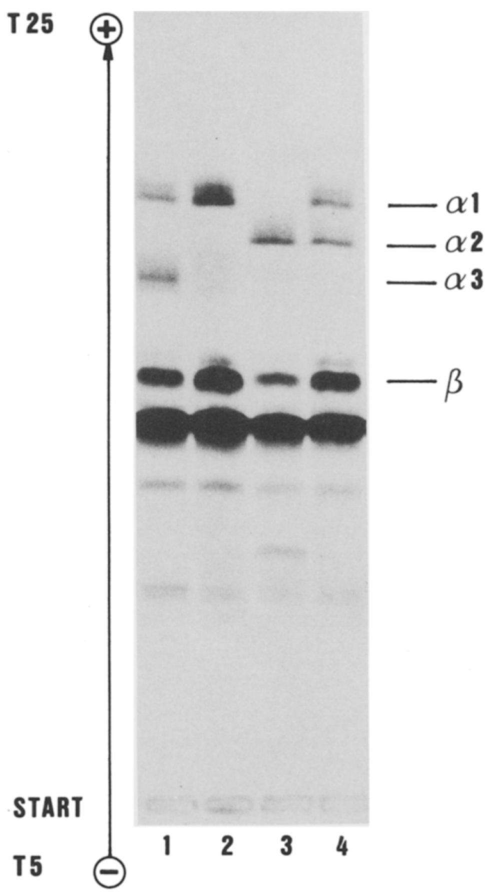
Fig. 4. Presentation of a hpα 2-variant after IEF. Phenotypes from left to right: Father (1) Hp 2FS-2FS, mother (2) Hp 2var-1S, first child (3) Hp 2FS-2var, second child (4) Hp 2FS-2var, third child (5) Hp 2FS-1S, fourth child (6) Hp 2FS-1S. (7): Reference sample Hp 1F-1F

Unequal but homologous crossing-over involving an intragenic recombination between two alleles Hp*2 gives rise to a triplicated α -chain gene called Hp Johnson. The Johnson phenotype has been observed in various populations in low frequencies and seems to vary slightly in its electrophoretical pattern (for review see Ritter et al. 1975). This phenotypic variation may be due to the 8 possible combinations of S- and F-parts of the α_3-polypeptide (Smithies et al. 1962). In this study four different persons (two of them kindly provided by Dr. Arnold, Augsburg) were typed Hp J-1 using starch gel electrophoresis. These results were confirmed by horizontal SDS-acrylamide gradient electrophoresis of isolated and reduced Hp.



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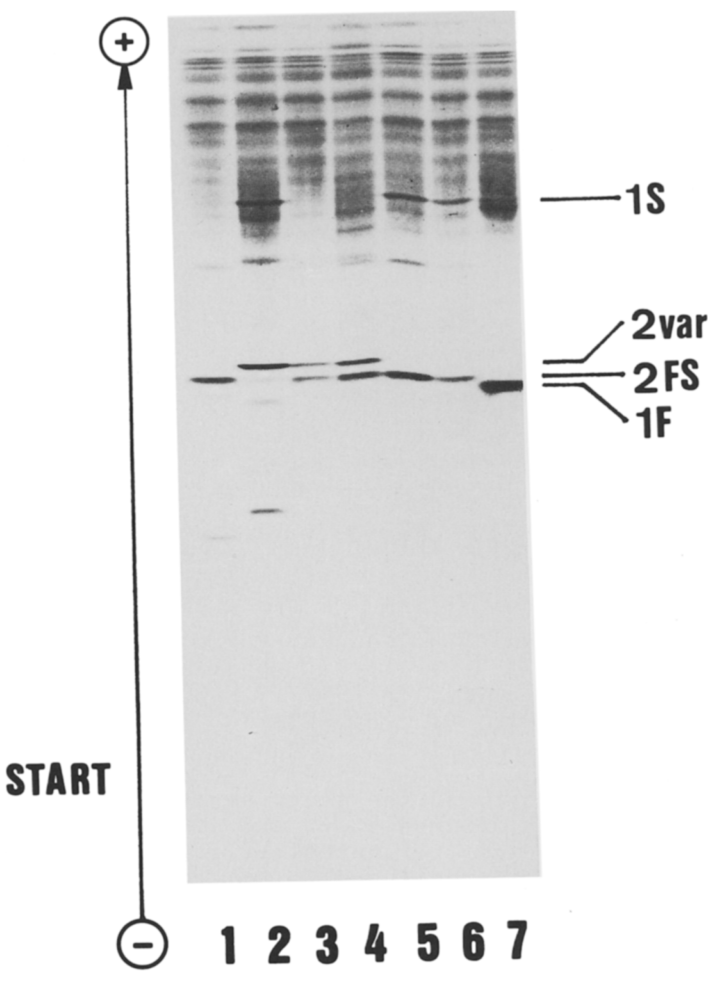
to separation quality. The hybrid Immobiline technique is therefore suitable to confirm hp 2FF-diagnosis after carrier ampholyte IEF without need of a second isolation step.

In addition to the main Hp phenotypes many variants both for the α and ß-chain genes were described, most of them recognizable after electrophoresis of native Hp (for review see Ritter et al. 1975, Bowman and Kurosky 1982). In this report an α2-chain variant, only recognizable by the subtyping method, is presented. Figure 4 shows variant Hp phenotypes observed in a Turkish family, as revealed by the IEF method. The father (1) was phenotyped to be Hp 2FS. The mother (2), pretyped Hp 2-1 by horizontal starch gel electrophoresis of native Hp, shows a gene product hp 1S and a second band anodal to the 2FS-position. This variant, which is according to the pretyping result an α2-variant, was transmitted to the first (3) and second (4) child.



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One technical problem in Hp subtyping using this method is the sometimes unsatisfactory resolution of hp 2FF. Overloading effects near by the application point can lead to smeary and weak hp 2FF bands and therefore favour mistyping. To overcome this a technique of hybrid isoelectric focusing was tried: Polyacrylamide gels of the same size as above were cast with a linear immobilized pH range 5-7 as recommended by the supplier. Carrier ampholytes pH 5-7 (LKB) were added directly to the gel solution to give a final concentration of 0.5%. After polymerisation gels were run for 6 hours without prefocusing applying 12W, 4mA and 3000V. 25 mmol Glu/Asp and 0.1 mol NaOH were used as electrode solutions. Sample treatment, loading and staining were done in the same way as for the carrier ampholyte IEF gels. 9 different phenotypes as revealed by IEF in hybrid Immobiline gels are demonstrated in Fig. 3. Samples were first separated in a carrier ampholyte IEF gel, frozen and thawed next day for focusing in the hybrid Immobiline gel.

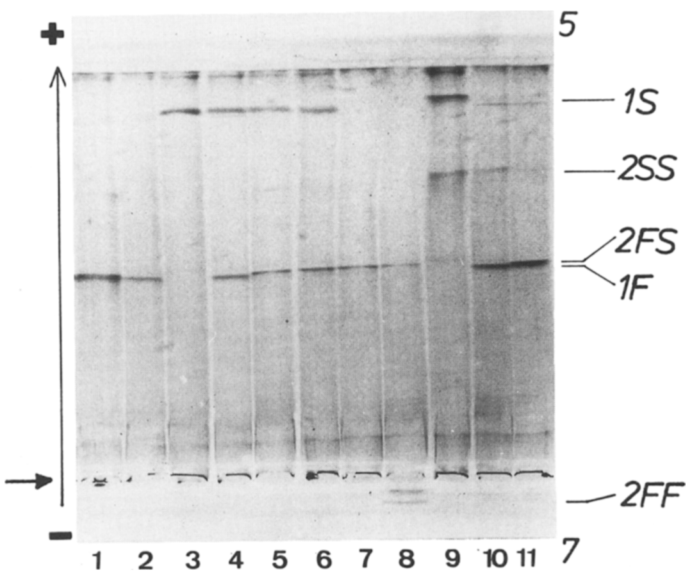

Fig. 3.Hybrid IEF separation of Hp chains. Phenotypes from left to right: (1) Hp 2FS-1F, (2) Hp 1F-1F, (3) Hp 1S-1S, (4) Hp 1F-1S, (5) Hp 2FS-1S, (6) Hp 2FS-1S, (7) Hp 2FS-2FS, (8) Hp 2FS-2FF, (9) Hp 2SS-1S, (10) Hp 2SS-1F, (11) Hp 2FS-1F

Due to the linear pH gradient and the short separation distance of 125 mm, gene products hp 2FS and hp 1F are worse separated in this gel as compared to the carrier ampholyte generated pH gradients. The advantage of this technique is the improvement of hp 2FF-typing. As can be seen in sample (8), hp 2FF is the only gene product that migrates cathodally together with an additional band. Both bands are sharp and clearly recognizable. One freeze-thawing cycle of treated samples does not seem to be detrimental

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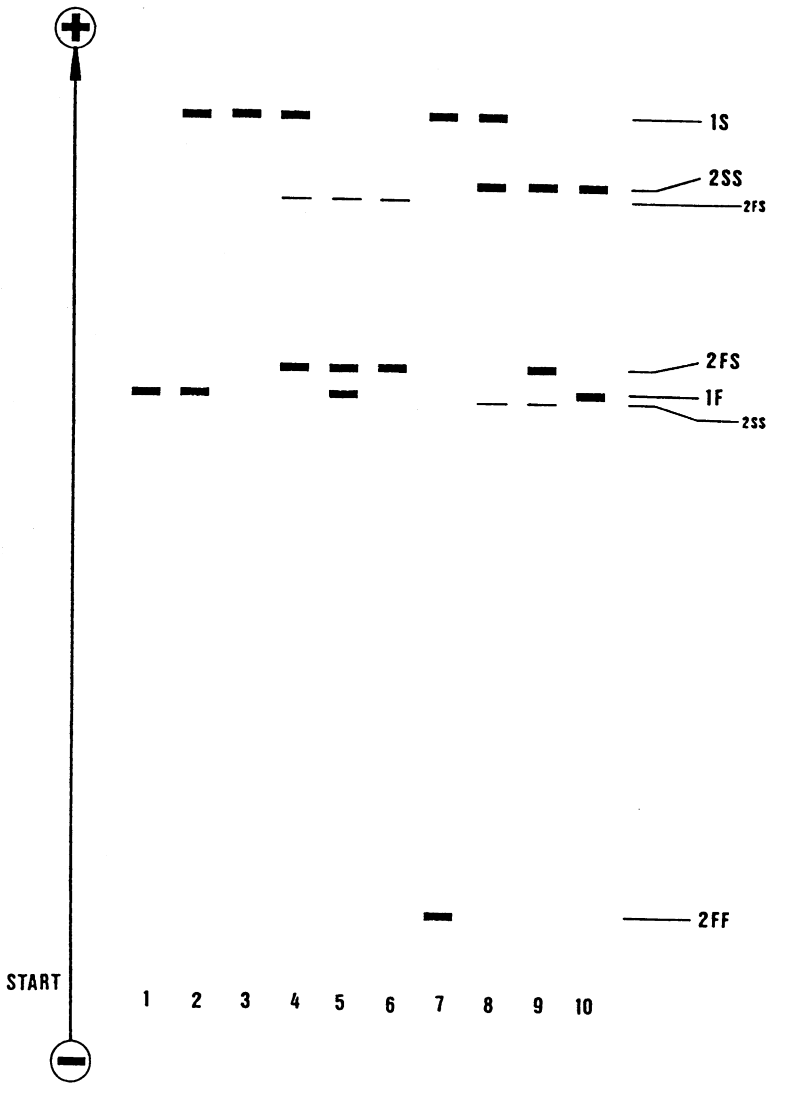

Fig. 2.Diagrammatical representation to Fig. 1. Major gene products hpα are drawn as strong bands

Analyzing 91 families with 268 children from Southwestern Germany the following gene frequencies were obtained: Hp*1F: 0.144, Hp*1S: 0.254, Hp*2FS: 0.574, Hp*2SS: 0.024, Hp*2FF: 0.004. These values are in good agreement with gene frequencies established in other European countries with the IEF method (Shibata et al. 1982, Patzelt and Schröder 1985) and show only slight deviation in Hp*1F-frequency calculated for a Norwegian population sample (Teige et al. 1985). Regarding this study the a priori exclusion chance of the Hp system raises to 28%.

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and Hp 2FF-1F, that are not represented on this picture, were also observed.

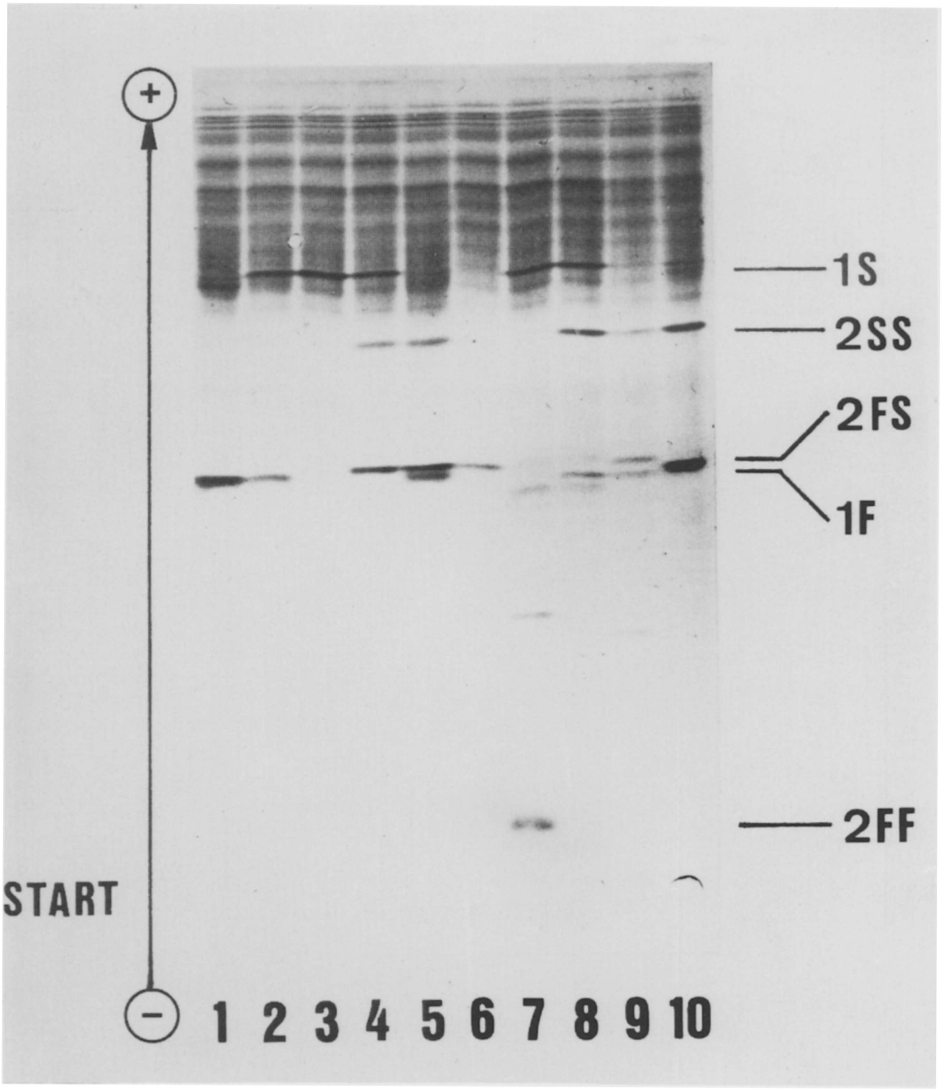

Fig. 1.Hp phenotypes (1) Hp 1F-1F, (2) Hp 1F-1S, (3) Hp 1S-1S, (4) Hp 2FS-1S, (5) Hp 2FS-1F, (6) Hp 2FS-2FS, (7) Hp 2FF-1S, (8) Hp 2SS-1S, (9) Hp 2FS-2SS, (10) Hp 2SS-1F as revealed by IEF of reduced and alkylated Hp

In Fig. 2 the subtype patterns of Fig. 1 are diagrammatically represented. Faint bands indicate the frequently occurring minor bands of gene products hp 2SS and hp 2FS.

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Subtyping of Hp: Common and rare phenotypes

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INTRODUCTION

Many techniques for Hp subtyping on protein level have hitherto been described (Connell et al. 1962, Pastewka et al. 1973). They either combine a purification step with subsequent electrophoretical separation and protein staining or include Hp identification by peroxidase staining (Linke 1984) or immunoblotting (Teige et al. 1985). Electrophoresis of reduced and alkylated Hp suffered from an unsatisfactory separation of the gene products hpα2. This was greatly improved by Shibata and coworkers (1982), who introduced an IEF technique, that clearly separates the gene products hp 2FS (SF), hp 2SS, hp 2FF, hp 1F and hp 1S. This IEF technique together with a batchwise ion exchange chromatography was used in this work to investigate gene frequencies of Hp subtypes in a Southwestern German population. Additionally separation of Hp chains in hybrid IEF will be presented and discussed. Furthermore a Hpα2-variant and two different Hp Johnson subtypes will be described.

MATERIALS AND METHODS

Purification and subsequent reductive cleavage of Hp was done as described previously (Zischler et al. 1987). 250 x 125 x 0.5 mm acrylamide gels (T5%/C3%) were cast with a final concentration of 0.8% 5-7, 0.8% 3-10 and 0.4% 6-8 Ampholine carrier ampholytes (Shibata et al. 1982). Maximum settings were 1500V, 25 mA and 5W. 1 mol phosphoric acid and 1 mol NaOH were used as electrolytes. Samples were applied cathodally on the prefocused gel and run until the presetted voltage was reached. Staining was done according to Blakesley and Boezi (1977).

RESULTS AND DISCUSSION

Figure 1 shows 10 Hp phenotypes as revealed by IEF of reduced and alkylated Hp. In our family material the phenotypes Hp 2FS-2FF

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DISCUSSION

The haptoglobin subtyping system has proved a reliable and efficient method for use in cases of disputed paternity. Only small amounts of serum is needed, and typing is achieved even in samples subjected to a high degree of haemolysis. The sensitivity of the method is very dependent on the antibody quality, and testing of antibody preparations, including different batches, from different commercial sources has been necessary. The antibodies presently in use in our laboratory have worked satisfactory for a long period of time.

The observed α 2-variant and the acid α 1-variant differ from normal 1 and 2 in isoelectric points only, and these variantions may be explained by single point mutations leading to an amino acid change in the peptides. The mechanisms behind the 1S-variant showing two peptides with different molecular weight, are not that easily explained. Further studies, preferrably on DNA level, might give informations on the genetic events leading to this variant.

The haptoglobin phenotype distribution found in the present material corresponds to Hardy Weinberg expectations, and the haptoglobin allele frequencies found in Norway are quite similar to the frequencies reported from other European countries.

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The β-chain variant described earlier (Teige 1986) appears with a slightly higher frequency than Hp 3 (Johnson). We have seen the variant in 5 unrelated individuals, and also in mother and child.

Paternity exclusion efficiency

The paternity exclusion efficiency of the system is evaluated in a material of 507 men involved in paternity cases and excluded in other systems than haptoglobin. Of these 507 non-fathers 163 were also excluded by haptoglobin subtyping, which is in agreement with the expected exclusion efficiency of the system, table 3.

The total exclusion efficiency of the other routine systems used in cases of disputed paternity in our laboratory is approximately 92 percent, and the 507 excluded men therefore represent only 92 percent of the non-fathers in our material. Thus, 14 single exclusions with the haptoglobin subtyping system is expected, which is in very good agreement with the 18 observed.

Table 2. Haptoglobin allele frequencies in 3318 unrelated individuals involved in cases of disputed paternity

HP ALLELE FREQUENCIES	1F	1S	2FS	2SS	2FF	3(J)
Shibata 1982 (France)	0.139	0.245	0.547	0.045	0.012	(N=202)
Thymann 1977 (Denmark)	0.16	0.27	0.54	0.03	0.002	(N=208)
Thymann 1987 (Denmark)	0.170	0.247	0.558	0.025	n.d.	(N=377)
Patzelt 1985 (GDR)	0.1471	0.2502	0.5753	0.0250	0.0020	0.0004 (N=1275)
Teige 1986 (Norway)	0.162	0.209	0.588	0.038	0.003	(N=606)
This material	0.160	0.226	0.572	0.037	0.004	0.0006 (N=3318)

n.d.= not determined

Table 3. Haptoglobin paternity exclusion efficiency in a material of 507 non-fathers[1]

	Observed	Expected
Number of Hp exclusions	163	165
Percent Hp exclusions	32.1	32.6

[1]Excluded in other systems

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intensity. One band has the same isoelectric point as normal 1S main band. The other band is more acidic, but a little less than the normal 1S minor band. The two-dimensional pattern of the variant shows two faint spots, fig. 2. One of the spots corresponds to normal 1S both in molecular weight and isoelectric point, but not in relative intensity to 2FS. The other spot is more acidic and heavier. As in the one-dimensional picture, the intensity of the two spots is equal.

Acid Hp 1-variant

An acidic α-chain variant is observed in a child and in the mother`s husband. The haptoglobin isofocusing pattern in serum from the child is 2FS1var., Fig. 1, lane 9 , and in serum from the husband 1S1var., Fig. 1, lane 10. The variant's main band has the same isoelectric point as the acidic minor band of 1S, and the variant's minor band is correspondingly more acidic. Two-dimensional SDS electrophoresis of the variant confirms its isoelectric point, and shows that the variant alpha peptide has the same molecular weight as 1F and 1S.

Phenotype distribution and allele frequencies

The haptoglobin phenotype distribution in 3318 unrelated individuals examined at the Institute of Forensic Medicine in Oslo, is presented in table 1. The allele frequencies determined in this material and in an earlier Norwegian material, are presented in table 2. Haptoglobin allele frequencies in Denmark, France and GDR are included in the table for comparison.

Hp-types	Observed	Expected
1S	183	170.0
1F	79	85.0
1F1S	240	240.4
2FS	1076	1086.4
2FS2SS	156	140.0
2FS2FF	16	14.4
2SS2FF	1	0.9
2SS	4	4.5
2FF	0	0.05
2FS1S	841	859.6
2FS1F	628	607.8
2SS1S	50	55.4
2SS1F	30	39.2
2FF1S	3	5.7
2FF1F	5	4.0
3(Johnson)	0	0.001
32FS	2	2.3
32SS	0	0.2
32FF	0	0.02
31S	1	0.9
31F	1	0.6
2FS1Svar	1	0.6
1S1Svar	1	0.2
N	3318	3318.2

Table 1. Haptoglobin (HPA) phenotype distribution in 3318 unrelated individuals involved in cases of disputed paternity.

$chi^2 = 7.528$
d.f.=10
$0.70 > P > 0.50$
(Phenotypes with numbers less than 6 are counted together)

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RESULTS

In Fig. 1 is shown the isofocusing pattern of rare and common Hp -chain variants. The method offers easily recognizable patterns of each subtype, of which the more common subtypes have been described earlier (Teige 1985, 1986). The Hp 3 (Johnson) has a 3 band pattern with one major and two minor bands. These bands are seen between the 2FS main band and the β- chain, Fig. 1, lanes 1 and 2. The Hp 2FF has a main band which are just a little more acidic than the β-chain, Fig. 1, lane 3.

HP 2-variant
The variant was observed in a blood sample from a suspect in a case of burglary. The Hp 2-variant has a slightly more acidic isoelectric point than 2FS, Fig. 1, lanes 4 and 5. This is most clearly seen in Fig. 4, lane 4, which shows a mixture of normal 2FS and the 2-variant. Two-dimensional SDS electrophoresis confirmed the variant's isoelectric point, and showed that the variant has the same molecular weight as normal 2FS.

Hp 1S-variant
This variant was observed in a child and in the alleged father. The alleged father is typed as 2FS1Svar, the child as 1S1Svar and the mother as 2FS1S, Fig. 1, lanes 6, 7 and 8. The variant's isofocusing pattern shows two bands of equal, but rather low

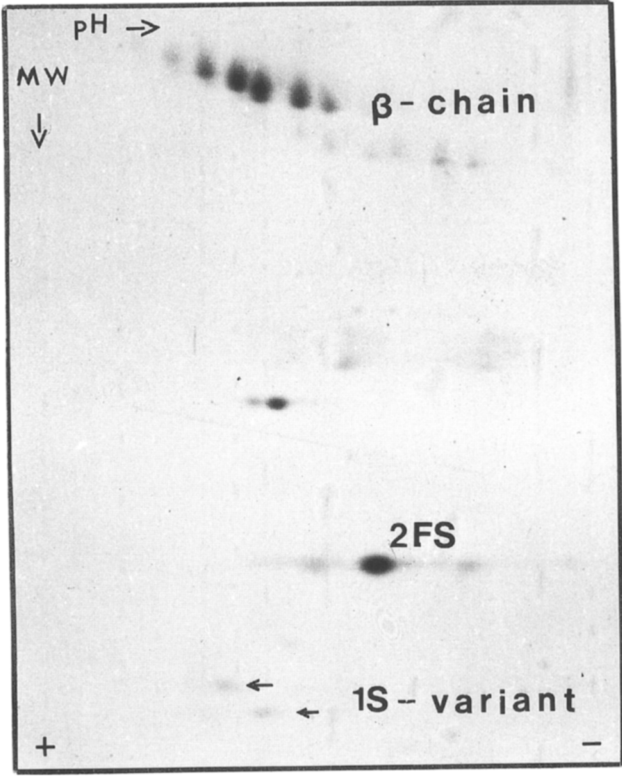

Figure 2.
Two-dimensional SDS electrophoresis of serum haptoglobin immunoprecipitate from alleged father showing the 1S-variant. The alleged father is typed as 2FS1Svar.

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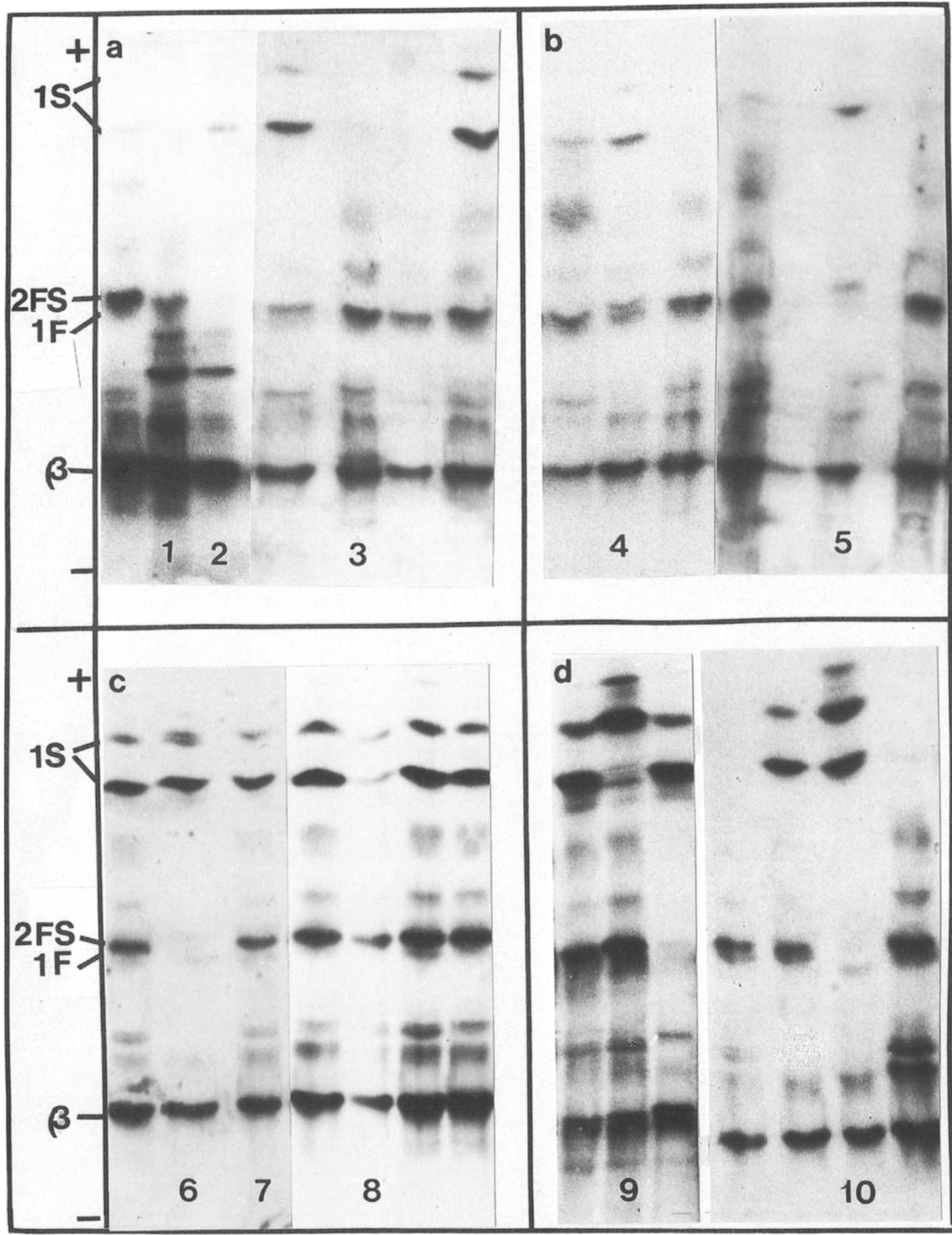

Figure 1. Isofocusing patterns of common and rare haptoglobin α-chain variants. Lane 1: 31F(mother) Lane 2: 31S(child) Lane 3: 2FS2FF Lane 4: Mixture of normal 2FS1S and 2var1S Lane 5: 2var1S Lane 6: 2FS1Svar(alleged father) Lane 7: 1S1Svar(child) Lane 8: 2F1S(mother) Lane 9: 2FS1var(child) Lane 10: 1S1var(mother's husband)

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Patterns and frequencies of common and rare Hp α-chain variants

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INTRODUCTION

The α-chain polymorphism of the serum protein haptoglobin (Hp) has for a long time been used in cases of disputed paternity. The most common Hp α-chain alleles are Hp1 and Hp2, which by further subtyping can be divided into Hp1S, Hp1F, Hp2FS, Hp2SS and Hp2FF.

In cases of disputed paternity conventional Hp typing offers an exclusion efficiency that is about half of the efficiency expected with Hp subtyping. But until a few years ago all available Hp subtyping methods required a purification step before the electrophoretic separation and identification could be done, and in most laboratories routine subtyping of haptoglobin was not performed.

An isofocusing/immunoblot method, requiring no prior purification of the Hp molecule (Teige 1985), has from 1985 been used routinely at the Institute of Forensic Medicine, Oslo, in all cases of disputed paternity as well as in some cases of criminal investigation. During this period one new β-chain variant and three new α-chain variants have been detected. The β-chain variant has already been described (Teige 1986), and the α-chain variants will be shown here. We will further give the Hp phenotype distribution and the allele frequencies obtained by subtyping 3318 unrelated individuals. The exclusion efficiency of the haptoglobin subtyping system is also evaluated.

METHOD

The method has previously been described in detail (Teige 1985).
The following antibodies are now used:
Anti-human haptoglobin produced in rabbit, from Dakopatts A/S, P.B. 1359, 2600 Glostrup, Denmark.
Anti-rabbit IgG conjugated to alkaline phosphatase, produced in swine, from Orion Diagnostica, Espoo, Finland.
The alkaline phosphatase staining is performed according to Blake (1984).

Molecular weights and isoelectric points of variants are determined with two-dimensional SDS electrophoresis as described by Olaisen (1981).

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Table 1. Hp phenotype distribution among 1035 unrelated individuals and corresponding gene frequencies

Phenotype	Observed n	Observed %	Expected n	x^2	allele frequencies
1F -1F	19	1.84%	20.03	0.05	
1F -1S	74	7.15%	74.16	0.00	
1S -1S	69	6.67%	68.62	0.00	Hp*1F = 0.1391
1F -2FF	0	0.00%	0.42	0.42	*1S = 0.2575
1F -2FS	172	16.62%	167.93	0.10	*2FS = 0.5831
1F -2SS	4	0.39%	5.43	0.37	*2SS = 0.0188
1S -2FF	0	0.00%	0.77	0.77	*2FF = 0.0014
1S -2FS	309	29.86%	310.79	0.01	
1S -2SS	12	1.16%	10.04	0.38	
2FF-2FF	0	0.00%	0.00	0.00	
2FF-2FS	3	0.29%	1.75	0.89	
2FF-2SS	0	0.00%	0.06	0.06	
2FS-2FS	350	33.82%	351.90	0.01	
2FS-2SS	23	2.22%	22.74	0.00	
2SS-2SS	0	0.00%	0.37	0.37	

Sum of Chi-square for (df 10)=3.44 (0.975>p>0.95)
(df 5)=0.97 (0.975>p>0.95)

Table 2.Hp allele frequencies of different studies

Study	n	Hp alleles *1F	*1S	*2FS	*2SS	*2FS
Bertrams et al.1987	1035	0.1387	0.2538	0.5864	0.0196	0.0015
Thymann et al.1977	208	0.16	0.27	0.54	0.03	0.00
Oleisen et al.1981	52	0.13	0.21	0.63	0.03	0.00
Shibata et al.1982	202	0.1421	0.2462	0.5533	0.0457	0.0127
Patzelt and Schröder 1986	1275	0.1471	0.2502	0.5753	0.0251	0.0020
Teige et al. 1986	606	0.162	0.209	0.588	0.038	0.003
Zischler et al.1987	182	0.144	0.254	0.574	0.024	0.0004

DISCUSSION

The excellent agreement of Hp allele frequencies obtained by different groups especially among the same population demonstrates the usefulness of Hp subtyping for paternity testing. No single deviation for expected Hp subtypes according to results obtained by SGE was observed among more than 200 matings. The exclusion chance of the Hp system increases from 18% by SGE to 33% by IEF. This is the highest exclusion chance of all IEF systems as shown in Table 3.

Table 3. Exclusion chance of IEF systems(Mendner and Kühnl 1986)

System	Exclusion chance	System	Exclusion chance
Hp	33%	C8	19.27%
PGM1	31.91%	C6	18.77%
GC	29.74%	A2HS	17.81%
PI	29.29%	FUCA	15.10%
GDH	26.90%	ESD	9.77%
PLG	22.58%	AMY2	4.52%
F13B	22.29%	C2	2.83%
TF	19.43%		

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equipment (Ultrophor, Macrodrive 5, Multitemp II). Before polymerization an ampholine mixture (Ampholine LKB, two parts pH 3.5-10, two parts pH 5-7. one part pH 6-8) is added in 5% proportion of the total gel (0.8ml carrier ampholytes for a 16ml gel). Gels can be stored at 4-6°C up to a fortnight. Electrode paper strips for prefocusing and focusing were soaked with 0.5M NaOH for the cathode and with 0.5M H3PO4 for the anode. After prefocusing of the gel for 30min. at 5-10°C samples were applicated on to the gel by applicator pieces (Whatman No1, 5x10mm). IEF was performed for 150min. Applicator pieces were revomed after 30min. Maximum electric values for prefocusing and focusing were 1600V, 10mA and 10W. Fixation, staining and decolorization of the gels were performed according to Steck et al. (1980).

RESULTS

Hp subtype patterns obtained by IEF of purificated and cleaved Hp proteins are presented in Fig.1. According to an isoelectric point of pI=6.52 the major band of Hp 2FF appears in our technique in the very cathodal region of the gel near the sample application area.

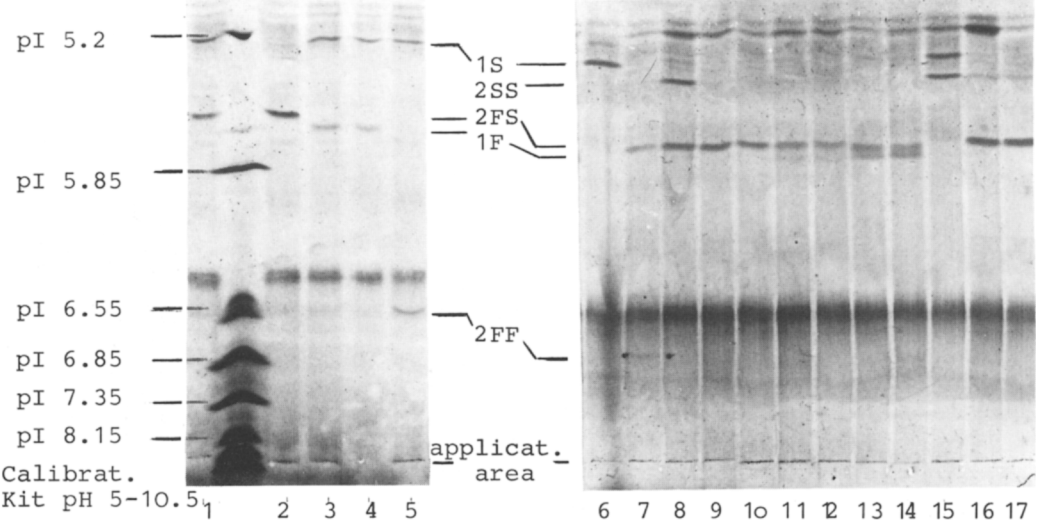

Fig. 1. Hp subtypes from left to right: 1) 1S-2FS 2) 2FS 3) 1S-1F 4) 1S-1F 5) 1S-2FF 6) 1S 7)2FS-2FF 8)2SS-2FS 9) 2FS 10) 2FS 11) 2FS 12) 2FS 13) 2FS-1F 14) 2FS-1F 15) 1S-2SS 16) 2FS 17) 2FS

Observed and exptected phenotype frequencies and the corresponding gene frequencies of Hp subtypes are presented in Table 1. There is no deviation of the observed from the expected values.

Comparison with allele frequencies obtained by other investigators including different techniques also yielded very good aggrement (Table 2).

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resulted from the unequal crossing over, Hp*2FF, *2FS and *2SS. Accordingly altogether five Hp alleles can be identified by IEF techniques.

The confirmation and polymerization of Hp gene products (α1,α2 and ß chains) is another point which has to be explained for a better understanding of the Hp system. After transcription and processing the Hp m-RNA initiats the translation of one large polypeptide chain, which contains the α and ß parts of the Hp molecule. These chains are cleaved by limited proteolysis to form an α-ß dimer. An individual, being homozygous for the Hp*1 gene, forms tetrameric molecules with two ß chains and two α1 chains. According to the alleles Hp*1F and *1S, both α1 chains carry the same or both allotypes. An individual being homozygous for the duplicated H*2 gene produces dimers with the larger α2 chains. Each of these chains carry Hp subtypes according to the composition of the Hp*1 genes (1F,1S), which had produced the Hp*2 gene by unequal crossing over, Hp*2FF, *2FS or *2SS. An individual being heterozygous for the Hp*1 and Hp*2 genes forms tetramers out of different dimers with short α1 and long α2 chains. According to free cysteine residues of the α2 chains Hp2 homozygote and Hp 2-1 heterozygote molecules tend to polymerize according to following formulas. Hp 2-2: (α2ß)•n,n= 3,4,5...; Hp 2-1: (α1ß)•2x(α2ß)•n, n=0,1,2...Multiple Hp bands seen in SGE are indicators of different degress of polymerization (Yang and Prybylska 1983, Pastewka et al. 1985).

MATERIAL AND METHODS*

Sera
Non hemolytic sera from 1035 normal, unrelated Caucasian blood donors of the German Rhine-Ruhr area.

Hp purification
100(200µl) of non hemolytic serum was added to 2ml(4ml) DEAE suspension which was prepared by mixing one part of a 1% aqueous DEAE stock solution with two parts of a 10mM sodium acetic buffer (pH 4.7). After centrifugation at 5000g for 5min. the clear supernatant was sucked off. To elute the hemoglobin molecules the pellet was washed with 2ml of the sodium acetic buffer and resuspended with 100µl (200µl) of a 0.125M ammonium acetic solution. After additional centrifugation for 10min. 40µl of the clear haptoglobin containing supernatant was transfered to a second tube for reductive cleavage.

Reductive cleavage
40µl Hp containing supernatant was mixed with 40µl of a reductive reagent, which was prepared by dissolution of 1g urea in 1.2ml borate buffer of pH 8.8 (0.1M boric acid, 0.04M NaOH) plus 20µl of ß-mercaptoaethanol. The mixture was incubated for 30min. and 8µl of an iodacetamide solution (92mg iodacetamide in 1ml of aqua dest.) was added to prevent refolding of the separated α and ß chains of the Hp molecule.

Isoelectric focusing and staining
IEF of the cleavage products was performed in polyacrylamide gels (T=5.5%, C=3%; 260x125x0,5mm) on gel bond films using the LKB

*A very detailed description of the method is availabe at request.

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Haptoglobin (Hp) subtypes in the German Rhine-Ruhr area

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INTRODUCTION

The two allelic polymorphism of the haptoglobin (Hp) system was detected by means of starch gel electrophoresis (SGE) more than 30 years ago (Smithies 1955). With an exclusion chance of 18% the Hp system has become a regular part of paternity testing since many years. Although Hp subtypes were discovered already in 1962 by Smithies et al., technical problems in producing unequivocal Hp pherograms of all five subtypes Hp1F,1S,2FF,2FS and 2SS hindered its common use in paternity testing. Only after application of isoelectric focusing (IEF) technique and after description of practicable techniques for purification of the Hp molecule (Oleisen et al. 1981, Santoro et al. 1982, Shibata et al. 1982, Patzelt and Schröder 1985) and after application of immunoblotting to Hp subtyping as well (Teige et al. 1983, 1986) all conditions for large scale routine Hp subtyping are given.

We present here Hp subtype frequencies determined in a large population from Western Germany (Rhine-Ruhr area) according to the method published by Patzelt and Schröder (1985), who worked out the technique of IEF of haptoglobin cleavage products. This method was developed by Shibata et al. (1982) and meanwhile investigated also by Zischler et al. (1987) in a Southwestern German population.

GENES AND PROTEINS

A short comment should be given to the rare and interesting phenomena of duplication of genes as well as confirmation and polymerization of gene products existing in the Hp system. The Hp molecules are composed of two kinds of polypeptide chains, α and ß chains. The individual differences (genetic polymorphism) are based on variations in the α chain, with the ß chain being everywhere the same. Three alleles at the Hp locus on chromosome 16 are responsible for three main kinds of α chains in all mammals. Two of the alleles, Hp*1F and Hp*1S, control α chains that differ from each other only by a single specific replacement of one amino acid (α1 chains). The third allele, Hp*2, differs from both Hp*1 alleles in a much more striking way. It is nearly twice as long as the Hp*1 gene and its gene product (α2 chain) shows to be a combination of two nearly complete α1 chains arranged in tandem sequence. The formation of the Hp*2 allele obviously was the result of a genetic "accident" (Smithies et al. 1962, Nance and Smithies 1962), in which an ancestral Hp gene of normal length underwent an unequal crossing over with its allele in the homologous chromosome but at a non homologous site. Since two Hp*1 alleles exist, Hp*1S and Hp*1F, three different Hp*2 alleles

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Table 1. Distribution of BF phenotypes and allele frequencies in the six populations

	Japanese	Chinese	Thai	Filipino	Indian	Hungarian
no.	325	48	72	74	126	219
F	6	1	2	6	12	2
FFb1	3	-	-	-	-	-
Fb1S	7	1	-	-	-	-
FS	82	6	18	32	48	54
S	226	37	52	36	63	135
SS07	-	-	-	-	3	19
FS07	-	-	-	-	-	2
F1S	-	-	-	-	-	4
S07	-	-	-	-	-	1
SSb3	-	-	-	-	-	2
Rare[a]	-	3	-	-	-	-
BF*F	0.1492	0.0833	0.1528	0.2973	0.2897	0.1370
BF*S	0.8339	0.8750	0.8472	0.7027	0.6984	0.7968
BF*Fb1	0.0154	0.01	-	-	-	-
BF*F1	-	-	-	-	-	0.0091
BF*S07	-	-	-	-	0.0119	0.0525
BF*Sb3	-	-	-	-	-	0.0046
BF*R[a]	-	0.0313	-	-	-	-

[a] F025BS, SS045, SS03

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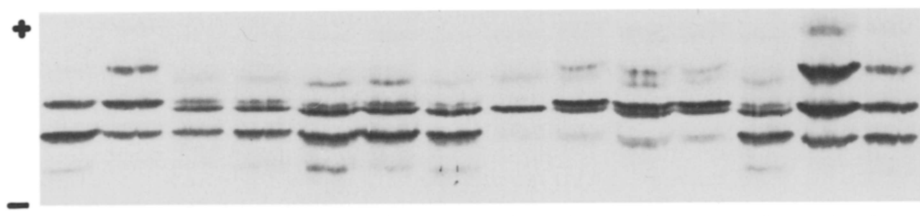

Fig. 2. Characterization of BF Fb1 by PAGIF. (1)S, (2)(14)FS, (3)(4)(12)Fb1S in Japanese, (5)(6)Fb1S in Chinese, (7)F025BS B:Bangkok, (8)Fb1, (9)(10)(11)FFb1, (13)F.

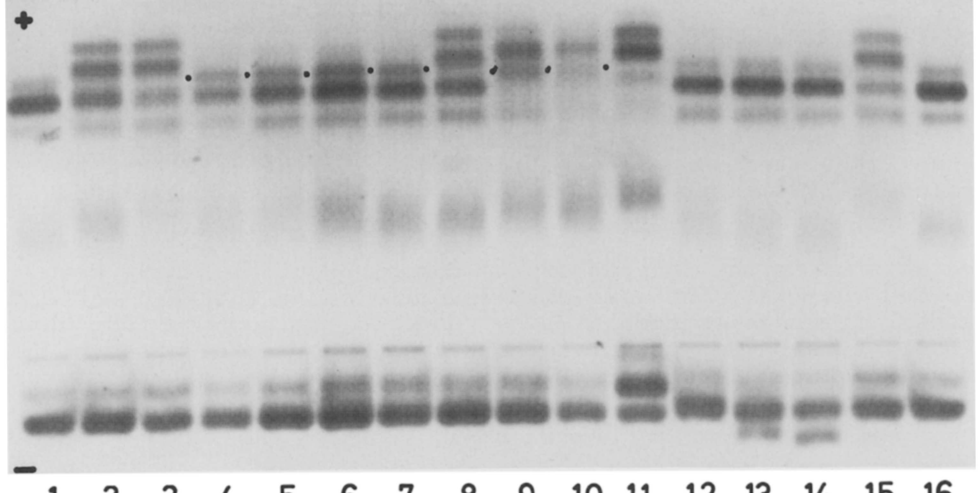

Fig. 3. Characterization of several BF phenotypes by AGE after zymosan treatment. (1)(16)S, (2)(8)FS, (3)(15)F, (4)Fb1S in Japanese, (5)(6)(7)Fb1S in Chinese, (9)(10)FFb1, (11)F075F, (12) F025BS, (13)SS03, (14)SS045. Dots indicate the Ba fragment of BF Fb1.

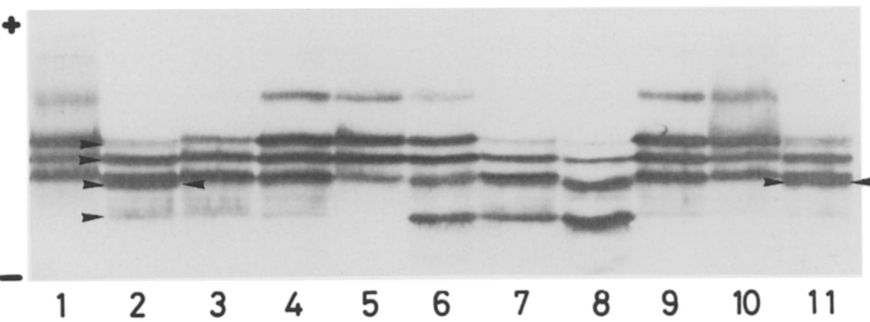

Fig. 4. BF patterns of Hungarian samples characterized by PAGIF. (1)(10)F1S, (2)(11)SSb3, (3)S, (4)(9)FS, (5)F, (6)FS07, (7)SS07, (8)S07. Arrows indicate Sb3 band.

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subdividing the BF*F allele into two subtypes which were termed BF*F and BF*Fb1, respectively. BF Fb1 exhibited a mobility slightly cathodic to BF F (Fig. 1), corresponding well to the pattern in PAGIF (Fig. 2). This subtypic pattern was observed only in Chinese and Japanese among the population groups of various ethnic origins. The allele frequency of BF*Fb1 in Chinese (n=88, additionally including 28 Chinese with psoriasis and 12 Chinese in Shanghai) and Japanese (n=325) was calculated at 0.017 and 0.0154, respectively. The serum samples carrying BF*Fb1 were treated with zymosan and then further characterized by AGE. The Ba fragment of BF Fb1 exhibited a different mobility, migrating between that of F and of S (Fig. 3). In addition BF*Fb1 was found to be firmly associated with C4 A3B2 haplotype. Although we have not extensively investigated on Negloid and Caucasoid populations except Indian and Hungarian yet using the method reported here, BF*Fb1 may be characteristic of some of Mongoloid populations.

In the Hungarian population a subtype of BF*S was detected at a low frequency by using PAGIF. This subtype seemed to be similar in the IEF pattern and allele frequency to BF*Sb1 reported by Weidinger (1984) and was designated BF*Sb3. The data obtained in this study were summarized in Table.

Several studies on subtypes of the common BF alleles have been reported thus far, but some discrepancies among subtypic patterns or their frequencies remain to be resolved. We propose thus that (1) all the subtypes postulated by each group are required to be compared with one another, (2) their serum samples must be further characterized by zymosan treatment, and (3) typing method for determining BF subtypes must be standardized.

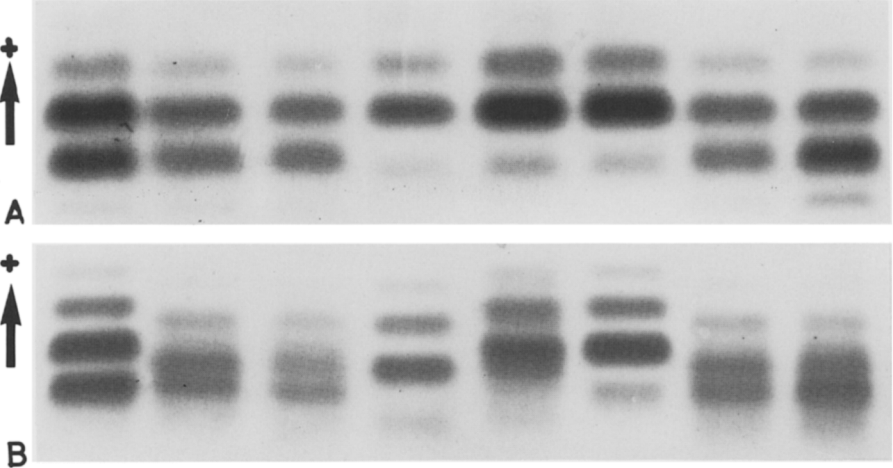

Fig. 1. Electrophoretic pattern of BF Fb1 defined by AGE/VB(A) and by AGE/TGVB(B). No difference in electrophoretic mobility is observed among the samples carrying BF*F by the standard VB but two types of BF F can be discriminated, one (Fb1) migrating a little slower to the other (F). From left to right; FS, Fb1S, Fb1S, Fb1, FFb1, F, Fb1S, Fb1S.

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Subtyping of Factor B by Agarose Gel Electrophoresis

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INTRODUCTION

Factor B (BF) polymorphism has been extended by the occurrence of subtypes in each of two common alleles, BF*F (Teng and Tan 1982; Geserick 1983; Abbal 1985; Nagai 1986; Nakamura 1987; Nishimukai 1987) and BF*S (David 1983; Weidinger 1984). Isoelectric focusing (IEF) has contributed to these studies, but there is a lack of correspondence among the subtypes postulated by each group or their frequencies. We present a BF F subtype using agarose gel electrophoresis (AGE) in Tris/glycine/Veronal buffer, further characterize them by zymosan treatment and by IEF in polyacrylamide gels (PAGIF) and also show a BF S subtype in the Hungarian population.

MATERIALS AND METHODS

EDTA-plasma samples were collected from 325 Japanese, 74 Filipino, 48 Chinese, 72 Thai, 126 Indians, and 219 Hungarian. Some serum samples were also collected for further characterization. Agarose gels (0.8%) were prepared both in the standard Veronal buffer (VB, Teisberg 1970) and in Tris/glycine/Veronal buffer (TGVB, O'Neill 1978). Constant voltage (20V/cm) was applied to gels till HbA marker migrated about 6 cm from the origin. Immunoprecipitated BF protein in the gels was stained with Coomassie Blue R250. Serum samples were treated with zymosan and then subjected to electrophoresis. PAGIF was done in a mixture of Ampholine (pH3.5-9.5, pH4-6, pH5-7) and BF bands were transferred to nitrocellulose filter by capillary blotting.

RESULTS AND DISCUSSION

AGE in TGVB could discriminate a subtype with a slower mobility from the previous type F defined by AGE in the standard VB, thus

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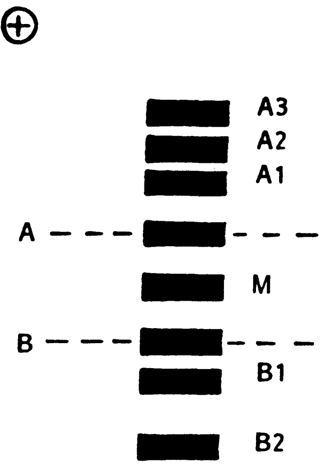

Abb. 1 zeigt die schematische Darstellung der Hauptbanden der C6-Allele nach Mauff et al. aus dem Jahr 1979.

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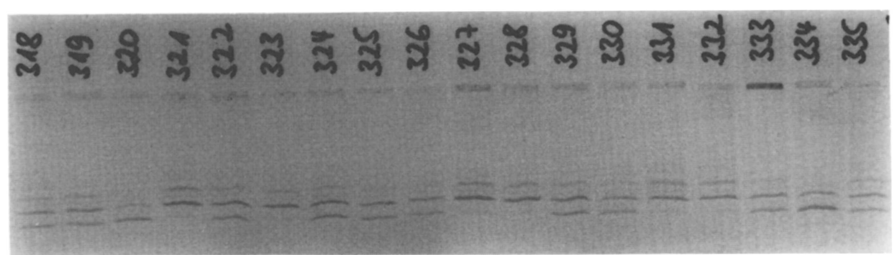
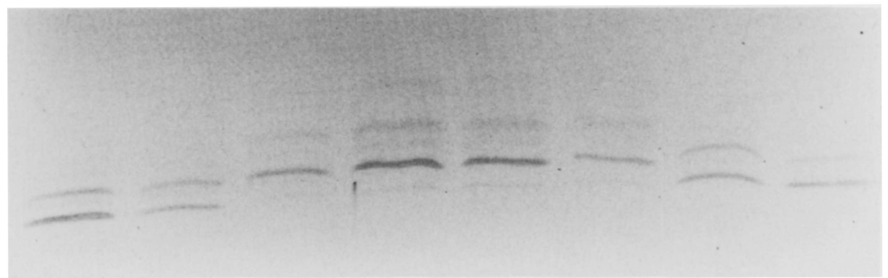
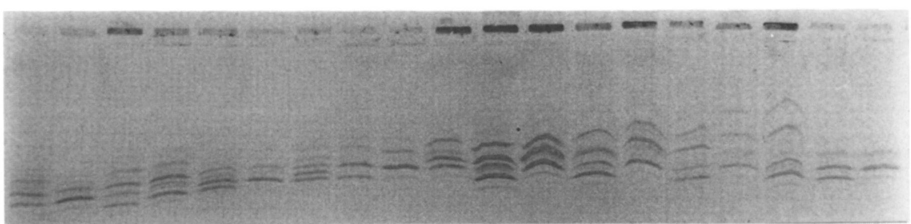


Folie Nr. 1 zeigt die häufigsten C6-Allotypen C6 A, C6 AB und C6 B mittels IAGIEF



Folie Nr. 2 zeigt zwei "unverträgliche" Mutter/Kind-Verbindungen.
Pos. 2 und 3 C6 B weak (Kd) / C6 A weak (Km);
Pos. 6 und 7 C6 A weak (Kd) / C6 B weak (Km).
In Pos. 1 und 4 befinden sich reinerbige C6 BB- bzw. reinerbige C6 AA-Kontrollen.
Der Dosiseffekt ist deutlich sichtbar.



Folie Nr. 3 C6-Varianten
von li. nach re. C6 AB, B, AB2, AB1, BB1, B, BM, AB, A, AA1, BA1, AA2, BA2, AA3, BA3, AA4, BA4, AB, A

Auf der Folie Nr. 3 sind alle in der Stichprobe gefundenen C6-Varianten dargestellt. In Pos. 8 und 9 von rechts sind A-Varianten zu sehen, deren Genprodukt schneller (anodisch) als A3 läuft: C6 BA>3 und C6 AA>3.
Wir haben diese Veriante vorläufig als C6 A4 bezeichnet.
Die Bestätigung einiger Varianten erfolgte durch Herrn Prof. Kühnl, Frankfurt, der uns auch freundlicherweise einige Referenzseren zur Verfügung stellte.

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Phänotyp	beobachtet		erwartet	
	n	%	n	%
A	458	39,25	453	38,81
AB	520	44,56	528	45,27
B	157	13,45	154	13,20
A1A	12	1,03	12	1,01
A1B	7	0,60	7	0,59
A-var	6	0,51	8	0,70
B-var	7	0,60	5	0,41
total	1167	100,00	1167	99,99

Genfrequenzen: C6*A = 0.6230
C6*B = 0.3633
C6*A1 = 0.0081
C6*var = 0.0056

$\chi^2 = 0.0211907;\ 0.99 < P(df=3)$
AVACH = 18,86 %

Tab. 1 C6-Phänotypen in Nordrhein-Westfalen

Die Tabelle 1 zeigt die Verteilung der in einer Stichprobe von 1167 nicht verwandten deutschen Personen aus Westdeutschland (Raum Nordrhein-Westfalen) beobachteten Phänotypen. Die errechneten Genfrequenzen stimmen recht gut mit anderen überein, die mit vergleichbaren Methoden ermittelt wurden. Die isolierte Vaterschaftsausschlußchance beträgt 18,86 %. Damit nimmt das C6-System unter den Isofokussierungssystemen bezügl. AVACH einen mittleren Platz ein.

Km \ Kd	A	AB	B	A1A	A1B	A-var	B-var
A	135	74		2		2	
AB	80	100	50			2	1
B		45	34				
A1A		5		4	1		
A1B		2		1	1		
A-var	2					1	1
B-var		2					
total	217	228	84	7	2	5	2

Tab. 2 545 Mutter-Kind-Paare

In Tabelle 2 werden 545 Mutter/Kind-Paare dargestellt, worin 292 "kritische" Verbindungen enthalten sind.

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DIE DARSTELLUNG DER HUMANEN C6-ALLOTYPEN DURCH IAGIEF

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Die Beschreibung des Polymorphismus der 6. Komponente des menschlichen Komplements erfolgte erstmals durch Hobart, Lachmann und Alper (1975). Die bisher weltweit veröffentlichten Genfrequenzen sind nur bedingt miteinander vergleichbar, da verschiedene Methoden angewandt wurden. Wir haben entsprechend der Darstellung der Untereinheit B des Gerinnungsfaktor 13 nicht Polyacrylamid, sondern Agarosegel verwandt.

METHODE

Gelherstellung: Die 0,5 mm dicken Agarose-Gele werden in einer Kassette auf GelBond Film (LKB 1850-101) gegossen. Für ein Gel (125x258x0,5mm) werden 0,16g Agarose IEF (Pharmacia 17-0468-01), 0,16 g Aces (Serva 10022) und 2,0 g D-Sorbit (Serva 35230) in 18,5 ml Aqua dest. im Wasserbad 10 Min. lang aufgekocht, gelöst und entgast. Die 0,9 ml Servalyt pH 6-7 (Serva 42925), 0,3 ml Ampholine pH 4-,65 (LKB 1818-116) und 0,6 ml Ampholine pH 5-8 (LKB 1818-126) werden nach dem Abkühlen auf 75°C dazugegeben. Das in die vorgewärmte Kassette gegossene Gel wird 1/2 Std. bei Raumtemperatur stehengelassen, dann für 1/2 Std. in den Kühlschrank gestellt. Das Gel wird aus der Kassette entnommen, mit einer Folie bedeckt und über Nacht in der Feuchtkammer im Kühlschrank gelagert.

Probenapplikation: 4µl Serum wird mit 4 µl Aqua dest. verdünnt und 4 Cm von der Anode mit Applikationsstreifen (Serva 42989) aufgetragen.

Isoelektrische Fokussierung: Mit einer Lage Filterpapier wird die Flüssigkeit von der Geloberfläche abgetupft.
Elektrodenlösungen: für die Kathode 0,25 mol NaOH, für die anode o,25 mol CH_3COOH.
Vorfokussierung: 30 Min. bei 1200 V, 50 mA, 8W (10°C).
Fokussierung: Nach der Applikation 30 Min. bei 150 V dann 180 Min. 1200 V, 50 mA, 8W und 10 Min. 1800 V.

Immunfixation: 500 µl monospezifisches Anti-C6-Serum (Cappel, USA 0101-1711) mit 200 µl Aqua dest. Wasser verdünnt wird auf dem Gel mit einem Glasstab verteilt und bei 37°C für 90 Min. inkubiert. Das Gel wird mit 2 Lagen Filterpapier, Glasscheibe und Gewicht 20 Min. abgepresst und über Nacht in 0,9 % Kochsalzlösung gewässert.

Färbung: Das Gel wird getrocknet und in 0,2 % Serva Blau R angefärbt; entfärbt. Färbelösung: 600 mg Serva Blau R, 120 ml Methanol, 30 ml Essigsäure, 150 ml Aqua dest.

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*A6 = 0,0296, *A4 = 0,0044, *A13 = 0,0044, *A5 = 0,0015; C4*B1 = 0,7101, *BQO = 0,1583, *B2 = 0,1139, *B6 = 0,0059, *B3 = 0,0044, *B5 = 0,0030, *Bvar = 0,0044.
Die hier vorgestellte Modifikation der kombinierten CPN/CPB-Vorbehandlung des C4 kann zwar den funktionellen Assay nicht ersetzen: Er weist eine höhere hämolytische Kapazität der C4-Genprodukte nach, die sich nach elektrophoretischer Trennung mit den "langsamen" C4A-Varianten überschneiden; dennoch ist die hier vorgestellte Modifikation ein vielversprechender Schritt in Richtung einer erweiterten MHC-Analyse durch die Erfassung sogenannter complotypes, welche neben den HLA-Antigenen auch C2, BF und die GLO umfassen.

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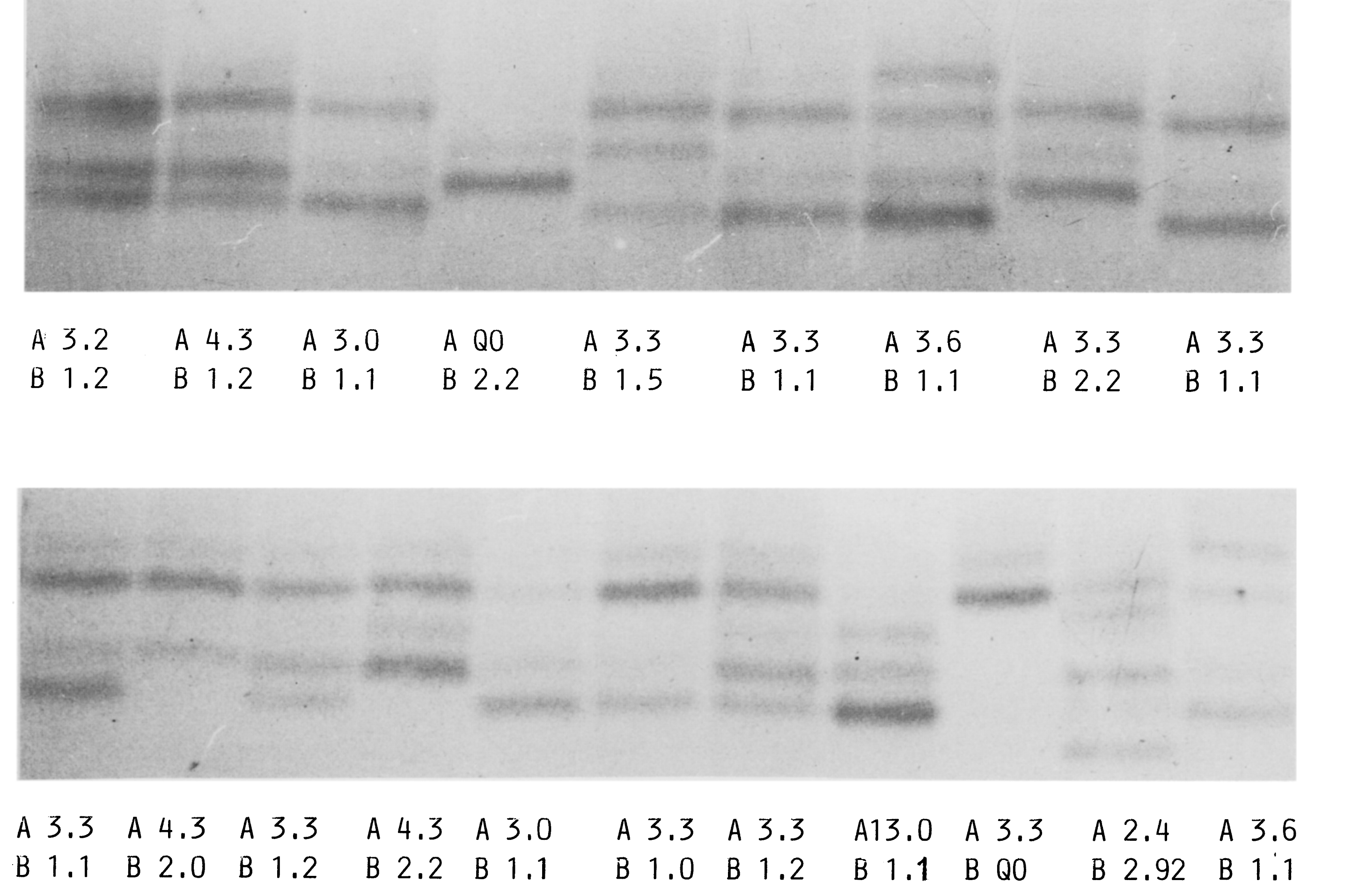

Abb. 3: Darstellung der C4-Phänotypen nach IAGE von CPN/CPB-behandelten EDTA-Plasmen

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Verhältnis zu den ursprünglich als erythrozytär determiniert angesehenen Blutgruppenmerkmalen Rodgers (Rg = C4A) und Chido (Ch = C4B) (O'Neill et al., 1978; Rittner et al., 1984). Diese beiden Blutgruppenantigen-Systeme sind identisch mit Strukturen auf der α-Kette der vierten Komplementkomponente (Tilley et al., 1978; Giles et al., 1984), welche sich nach Spaltung von C4 und Bindung der Spaltprodukte an Erythrozytenmembranen nachweisen lassen. Die von Middleton et al. (1974) nachgewiesene Koppelung zwischen Rg-, Ch- und HLA-Genen stellte somit eine vorweggenommene Entdeckung der C4-HLA-Koppelung dar.

Bemerkenswert ist weiterhin die unterschiedliche hämolytische Aktivität der beiden Proteine C4A und C4B. Unter gleichen Bedingungen zeigt das B-Protein eine beträchtlich größere funktionelle Aktivität gemessen am Hämolysegrad (Bitzan et al., 1983). Die biologische Bedeutung dieser Tatsache ist bisher noch unklar. Der Grund scheint jedoch auf molekularer Ebene zu liegen, denn die Bindung von aktiviertem C4B an Schafserythrozyten ist vierfach stärker als die von aktivierten C4A (Isenmann und Young, 1984).

Sim und Cross (1987) wiesen erstmals auf eine verbesserte Typisierungsmöglichkeit des C4 durch gleichzeitige Behandlung von EDTA-Plasmen mit Neuraminidase (Clostridium Perfringens Neuraminidase (= CPN Typ V oder VIII) und Carboxy-Peptidase B (= CPB-DFP) hin. Nach 3 h AGE (pH 8,9) mit anschließender Immunfixation sowie durch IPAGIF (2,5 bis 3,5 h, pH-Bereich 5 - 8) lassen sich die bislang komplexen C4-Bandenmuster deutlich vereinfachen (s. Abb. 1), so daß nur noch eine Haupt-Isoproteinbande pro C4-Strukturgen zur Darstellung kommt (Abb. 2 und 3). Auch eine Abschätzung der Heterozygotie für die häufigen stummen Gene beider Loci (C4A*QO und C4B*QO) war auf diese Weise möglich.

Insgesamt ergaben sich bei 368 nicht-verwandten gesunden Blutspendern aus Hessen sowie 15 Familien mit 36 Kindern Hinweise auf 42 verschiedene Phänotypen, die als Genprodukte von sieben C4A-Allelen und acht C4B-Allelen mit den folgenden Frequenzen interpretiert wurden: C4*A3 = 0,7885, *AQO = 0,1272, *A2 = 0,0444;

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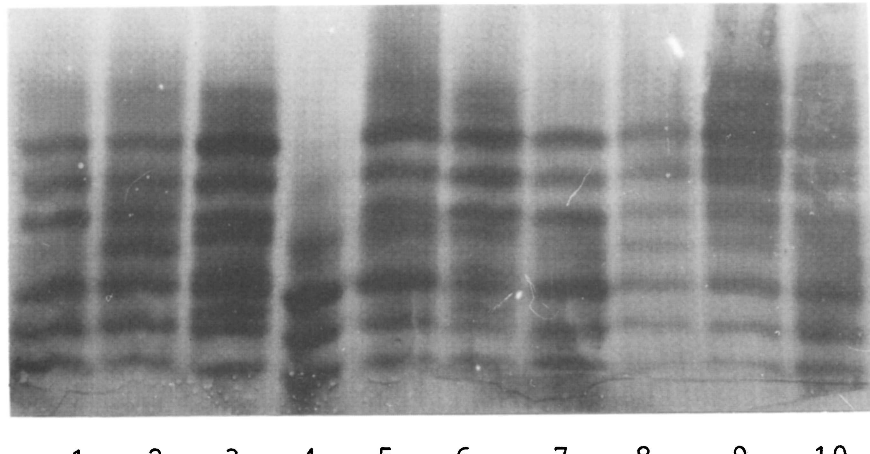

Abb. 1: Darstellungstechnik C4 durch IAGE mit Immuno-Blotting (Doxiadis, Essen, 1985)
Phänotypen: 1) A3 B1 2) A3 B1,2
3) A3 B1,3 4) AQO B1 5) A3 B1
6) A3 B1,3 7) A3 B1 8) A3 B1,2
9) A2,3 B1 10) A3 B1

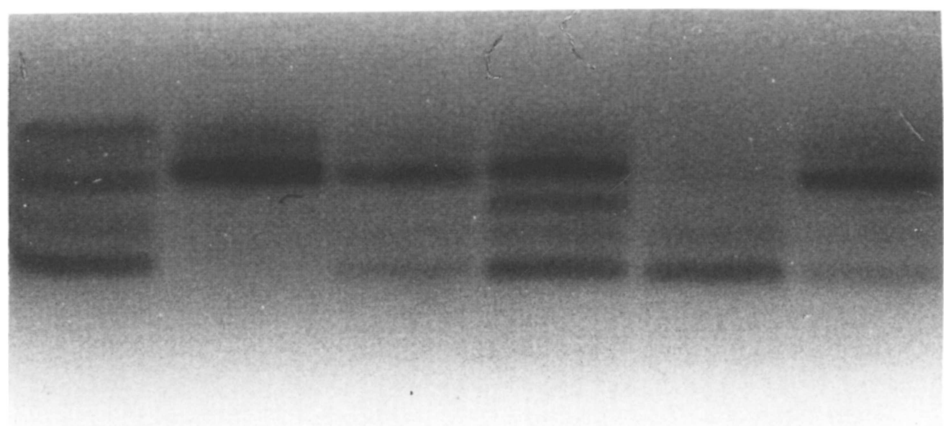

Abb. 2: Darstellung der C4-Phänotypen durch IAGE von CPN/CPB-behandelten EDTA-Plasmen (Genotypen-Schreibweise und Versuch einer Abschätzung von QO-Heterozygotie)
1) A3,QO B1,1 2) A3,3 BQO,QO 3) A3,3 B1,QO
4) A3,3 B1,6 5) AQO,QO B1,1 6) A3,3 B1,QO

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Verbesserte C4-Typisierung durch IAGE Neuraminidase- und Carboxy-Peptidase B-behandelter EDTA-Plasmen

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Ein genetisch determinierter Polymorphismus der vierten Komplementkomponente wurde 1969 zuerst von Rosenfeld et al. beschrieben. Methodische Verbesserungen zeigten einen außerordentlichen, komplexen Polymorphismus. Durch die Vorbehandlung der zu untersuchenden Seren mit Neuraminidase wurde es möglich, ein genetisches Modell des C4-Polymorphismus zu erstellen (Awdeh und Alper, 1980; Mauff et al., 1983). Man kennt heute mindestens zwei eng gekoppelte C4-Loci, jeder mit multiplen Allelen (O'Neill et al., 1978; Whitehead et al., 1984; Carroll et al., 1984). Auf Grund ihrer elektrophoretischen Auftrennungseigenschaften unterscheidet man ein Protein C4A, das anodennäher erscheint, da es mehr negative Ladungen trägt von einem Protein C4B, das langsamer wandert und kathodenwärts liegt. Nach der alten Nomenklatur wurden beide Proteine mit F (fast) und S (slow) angesprochen. Am Genort A sind mindestens 11 Allele, am Genort B mindestens 20 Allele bekannt (Mauff et al., 1983). An beiden Genorten sind mit unterschiedlich hoher Frequenz Nullgene zu finden (O'Neill et al., 1978).

Mauff et al. (1983) berichteten über einen genetisch determinierten Polymorphismus der β-Kette des menschlichen C4-Systems. Aus dem Pro-C4 werden 3 Ketten abgespalten. Eine davon ist die β-Kette mit 78.000 Dalton. Es wurden drei verschiedene C4-β-Kettenbandenmuster (H,HL,L) gefunden. Die beobachteten Phänotypen erfüllten jedoch nicht die Bedingungen des Hardy-Weinberg-Gleichgewichts für kodominante Allele.
Es konnten keine Beziehungen der β-Ketten-Phänotypen zu den α-Ketten-Phänotypen nachgewiesen werden. Ein Polymorphismus der menschlichen γ-Kette wurde bislang noch nicht beschrieben.
Beide Proteine, C4A und C4B zeigen ein sehr enges antigenes

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C3-SUBTYPES DEMONSTRATED BY "HOME-MADE IMMOBILINE" GENERATED IMMOBILIZED PH GRADIENTS AND IMMUNOBLOTTING. FREQUENCY ANALYSISES FOR HANNOVER AND LOWER SAXONY, FRG

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A convenient and considerably cost-saving method recently published by R. CHARLIONET & coworkers for preparing immobilized pH 5.5-5.7 gradients by using 0.2 m aqueous solutions of itaconic acid and dimethyl-aminopropyl-methacrylamide (DMAPMA) instead of commercially available Immobilines has been adopted to demonstrate subtypes of the third component of human complement by isoelectrophoresis and subsequent capillary immunoblotting on nitrocellulose.

The results of a population study for Hannover and Lower Saxony (N>500) including a calculation of the isolated paternity exclusion chance and an estimation of the parentage testing utility of the subtyped C3-system are presented.

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From the data presented before obviously C4 polymorphism will not be eligible for the paternity expertise, and little or moderate gain is to be expected from C2, C7, C4BP or factor I typing, whereas C5 and factor D are not polymorphic in most populations. More convenient methods in the future could well see factor H and C81 , due to their rather high single exclusion chances, as possible new candidates for the expertise in forensic haemogenetics.

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Table 3 : **Application of complement polymorphism**

	SENF (%)	technology[1)]	in forensic haemogenetics[2)]
C3	14.4	*	+
BF[3)]	14.6 (15.5)	*	+
C6	19.7	*	(+)
C81	20.8	***	(+)
C82	5.1	***	(+)
C4αA	24.3	***	-
C4αB	24.0	***	-
C2	4.9	**	-
C7	12.8	**	-
C5	6.1	***	-
C4BP	1.7	**	-
D	-	***	-
I	8.6	**	-
H	17.4	***	-

SENF : single exclusion chance for non-fathers
1) : * easy; ** moderately difficult; * difficult;**
2) : + commonly in use; (+) occasionally in use; - currently not in use
3) : in parenthesis including BF*F subtypes

From reported frequencies of polymorphic fragments single exclusion chances are in the range of those seen in protein polymorphisms. With the rapid explosion of experimental evidence from molecular genetics the data may not be complete. It appears, that however useful RFLPs have been for the fine characterization of the genome, in view of complex genetics and laborious technologies in forensic genetics they seem of limited value at present. It might therefore be expected that their application will remain for some time the domaine of a few specialized laboratories.

Reviewing the data on complement *protein* polymorphisms according to single exclusion chances, laboratory demand on technology and applicability to forensic haemogenetics (Table 3), they may be classified into three groups: *1.* polymorphisms with a high exclusion chance and frequent or occasional application in the expertise; *2.* proteins with complex genetics, variing exclusion chance and moderate or difficult demands on laboratory technology; *3.* polymorphisms with little gain in information or difficult technology. Among the two commonly used genetic systems, BF*F subtyping might potentially be introduced, as well as phenotyping of C6 on "Western blot" or immunofixation plates.

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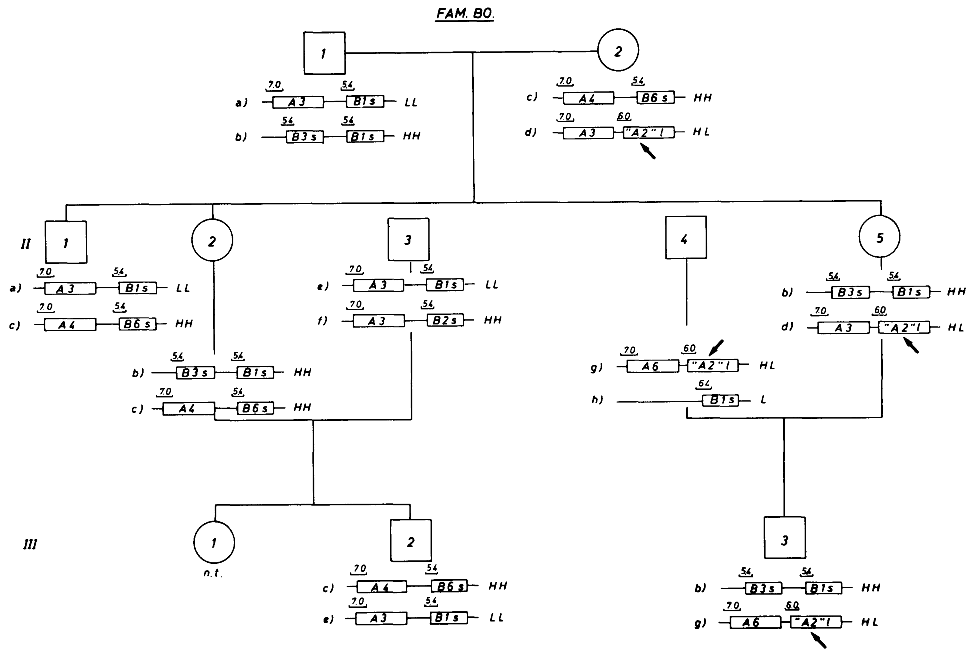

Figure 3 : **Pedigree of** ***Family BO*** **(for results of C4 phenotyping and RFLP cf. fig.2).**
Segregation of C4 genes according to C4 phenotyping, C2, BF, C4ß-chain (HH, LL, HL), and HLA data (not shown). Probe fragments (indicated above genes) recognize the 22 kb C4A gene (7.0 kb), the 22 kb long (l) C4B gene (6.0 kb fragment), the 16 kb short (s) C4B gene (5.4 kb fragment) and in individual II/4 the short C4B gene (6.4 kb fragment) in combination with a deleted C4A gene. (From: G. MAUFF, K. BENDER, M. BRAUN, M. BRENDEN, W. BOKSCH, E. DUTOIT, C.GILES, S. GOLDMANN, R. NEUMANN: C4-TaqI-RFLP in families with duplicated and aberrant C4 allotypes; in preparation)

This B-like A*2 seems also present in the A*6,A*2,B*Q0 haplotype g) in individuals II,4 and III,3.
In general it will therefore appear that C4 haplotypes with assumed deletions of one gene in fact may contain so called duplicated alleles in many instances which might have arisen from gene conversion (62). This may at length reduce the present estimate on silent and duplicated alleles but increase the frequency of aberrant genes. In addition, it must be mentioned that on the basis of Taq I-RFLP a considerable number of individuals seem to carry B*1 genes but do not express C4 protein (49).

Turning to the application of protein polymorphisms and RFLPs in forensic haemogenetics, as mentioned earlier, for three non-complement plasma proteins and five complement components RFLPs have been reported. They include *EcoR I*, *Hind III*, and *Pst I* polymorphism for HP, *BamH I* for DBP, and *EcoR I*, *Sph I*, and *Taq I* for PI (14). Restriction enzymes used for complement genes are listed in Table 2. The number of fragments obtained vary from two to a multitude of bands, such as in PI or C4, C4 having most extensively been investigated. Over all, *Taq I* RFLPs are most widely applied, although e.g. *BamH I* has proven to be useful in C4 and C2 RFLP (16), or *Bgl II* for the recognition of the C4A6 gene fragment (38) as well as for C3 RFLP (18). *Nlq IV* and *Eco0 109* in C4 distinguish only between C4A and B resp. Rg and Ch of the C4d fragment.

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proteins; *2.* the C4A*4,B*6 haplotype in conjunction with HLA-BW55,DRW6 on haplotype c) in individuals I,2, II,1, II,2, and III,2, B6 migrating in the C4A region; *3.* the linkage group HLA-B35/C4A*3,A*2,B*Q0 on haplotype d) in individuals I,2, and II,5, the duplicated "A2" obviously possessing the RFLP properties of a long 22 kb C4B gene; *4.* a second long C4B gene on haplotype g) as revealed in the RFLP, obviously coding for the so called "A*2" allele in duplication with A*6 in individuals II,4 and III,3; *5.* a short C4B gene on the C4A deleted haplotype h) seen in the RFLP of individual II,4 with the 6.4 kb intron as described by SCHNEIDER and coworkers (49).
From the most probable interpretation of results in this family it may be concluded that specifically the HLA-B35 associated duplicated A*3,A*2,B*Q0 haplotype contains a B-like A*2 gene, as first reported by PALSDOTTIR and coworkers (39) but in contradiction to a comparable haplotype recently described in family ST. by GILES and coworkers (25).

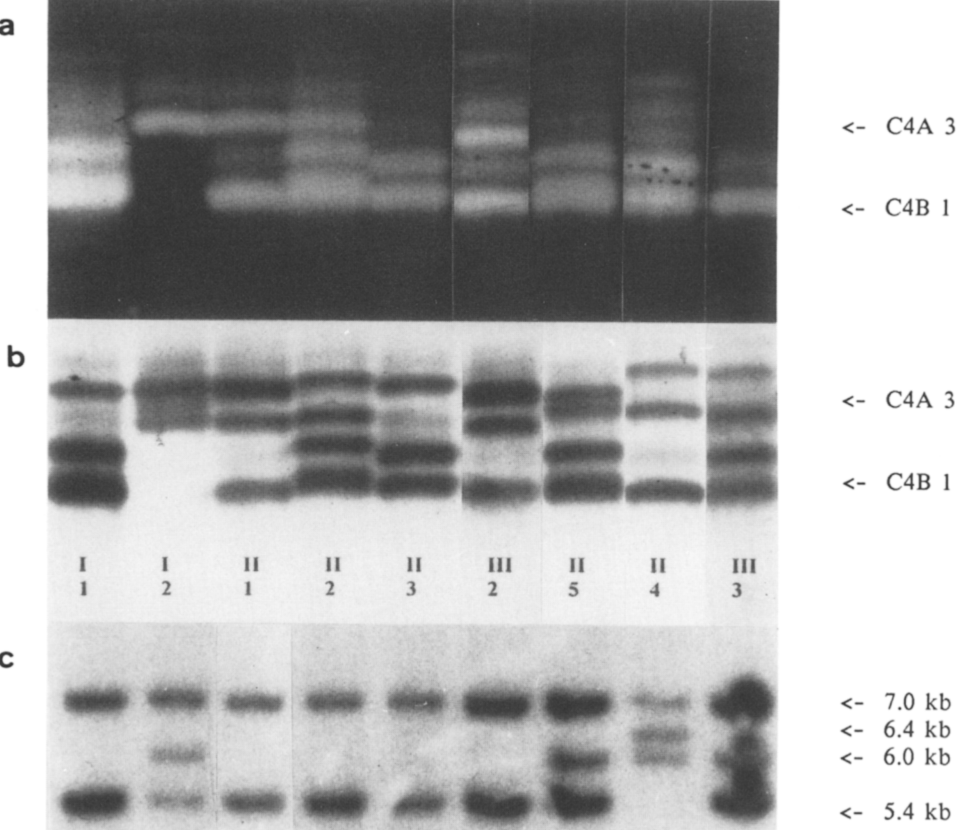

Figure 2 : C4 phenotypes and restriction fragment length polymorphism (RFLP) of *Family BO.* **For the designation of individuals and typing results cf. to pedigree in fig. 3. (a) Haemolytic overlay, (b) immunofixation with goat anti-C4 (ATLANTIC ANTIBODIES) after agarose-gel electrophoresis of carboxypeptidase B and neuraminidase treated samples (anode at top).**
(c) TaqI-C4-RFLP (anode at bottom); BamHI/KpnI fragment from full length cDNA-probe pAT-A (a gift from Dr. M. CARROLL, Boston).
(From: G. MAUFF, K. BENDER, M. BRAUN, M. BRENDEN, W. BOKSCH, E. DUTOIT, C.GILES, S. GOLDMANN, R. NEUMANN: C4-TaqI-RFLP in families with duplicated and aberrant C4 allotypes; in preparation)

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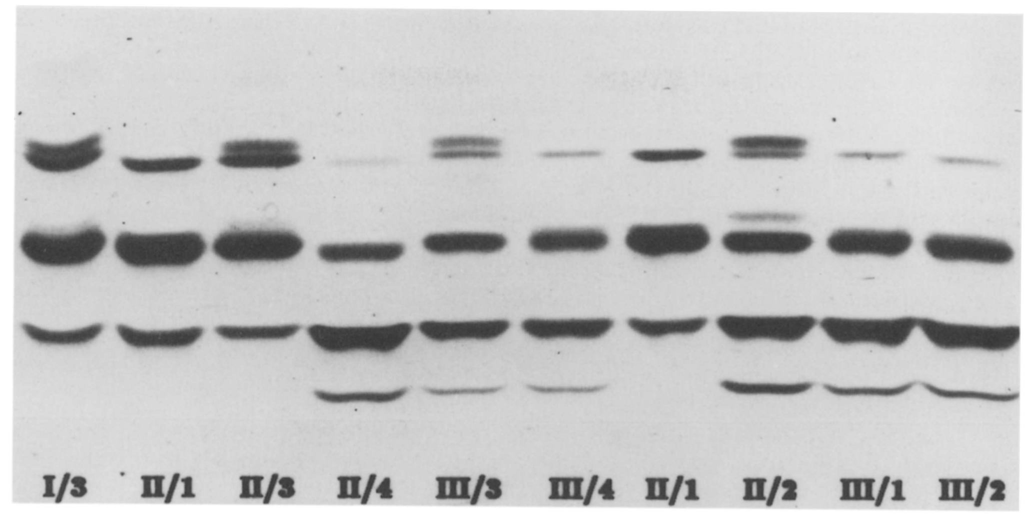

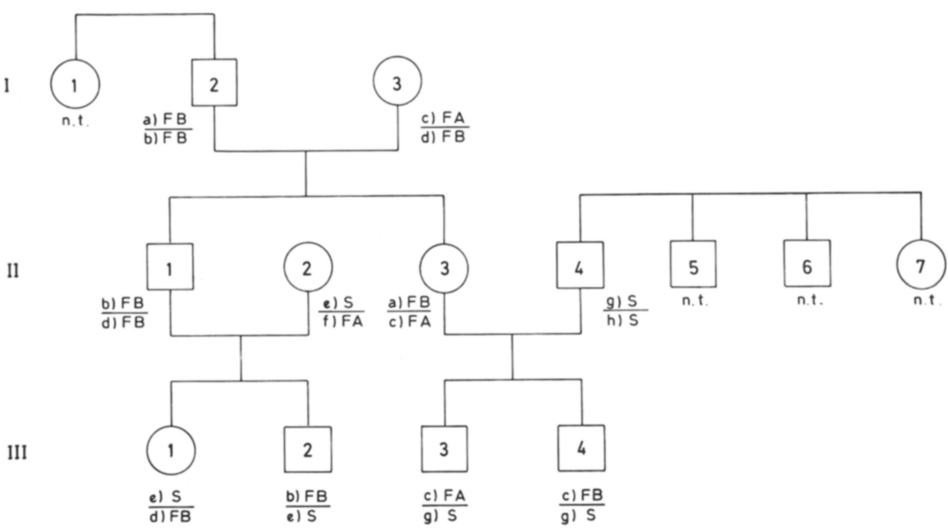

Figure 1: **BF*F subtypes : Segregation in *Family KLÖ.***

(a) Immunofixation of the factor Ba fragment with goat anti-B serum (ATLANTIC ANTIBODIES) after inulin treatment of samples, separation by agarose-gel isoelectric focusing, pH 4.5-5 (anode at top). Designation of individuals and BF phenotypes correspond to pedigree.
(b) Pedigree of Family KLÖ.
(From : G. MAUFF, I. SIEMENS, G. GESERICK, K. BENDER, G. PULVERER : The BF*F subtypes are detectable in the Ba fragment of factor B; in preparation).

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The amount of information on the major histocompatibility complex (MHC) class III proteins C2, BF, C4A, and C4B has reached a remarkable level. In the context of this review the available data may only be highlighted and the reader referred to more extensive reviews and original publications (4,7,8,9,34,44,45). Among the three class III proteins *C2* is least polymorphic with the common allele C and the rarer B and A alleles. *BF* polymorphism, consisting of the two common alleles F and S and a large collection of rare variants with different electrophoretic migration characteristics, has also been supplemented with the detection of subtypes (17,21,57). *C4* polymorphism, in short, consists of the two proteins C4A and C4B, which differ in general in their electrophoretic mobility, relative haemolytic activity in regard to their receptor binding properties (27), their antigenicity, and the size of their alpha chains in the reduced molecule. C4A*3 and B*1 are the most common alleles, A*6, A*4, A*2, B*3, and B*2 less common; a multitude of rare variants , exceeding nine for C4A and 18 for C4B, has been described (31,35).

Close linkage of class III components, presumptively having arisen by gene duplication is considered to reflect their structural similarities and/or functional interactions. The orientation of their genes between the MHC class I and II regions was long at variance (9). ABBAL and coworkers (1) through analysis of three unrelated French families with the very rare BF variant *S*11* have postulated the C4 genes to be oriented toward HLA-DR. Identical C4 haplotypes segregated with BF and HLA-A,B,C,DR,DQW in two families, but with a different C4 and DR haplotype in a third family. Assuming one common ancestral point mutation for the generation of *BF*S11* the recombination between BF and C4A must have been a later event. It would imply the following order of genes: HLA-B - C2 - BF - C4A - 21-OHA - C4B - 21-OHB - HLA-DR. This order has recently been confirmed through recombinant DNA methods (20).
Within the class III gene region also the structural genes for 21-hydroxylase (21-OHA and 21-OHB) have been located. The deficiency of 21-OHB is the cause of the salt-wasting form of adrenal hyperplasia (10). Furthermore, tumor necrosis factor (TNF), a lymphokine, has now been located within 260 kb to HLA-B (RAGOUSSIS, J., BLÖMER, K., WEISS, E., ZIEGLER, A., personal communication).

C2 phenotyping on the basis of functional assays, seemingly complicated to workers outside the complement field, has now been faciliated and made available to many laboratories using the "Western blot" technique which also detects phenotypes in the C2a fragment of decayed C2 (19,59).
Whereas phenotyping of BF S-subtypes was not successfully confirmed in most laboratories, doubts on the existence of BF F-subtypes are unjustified as shown in Figure 1 where segregation of BF*FA and BF*FB is demonstrated in a three generation family. In the Ba fragment of converted factor B distinction between BF FB with a single band and BF FA with one more pronounced anodal major and one cathodal minor band poses no problems; two bands of about equal strength are found in heterozygous FAFB carriers.
Phenotyping of C4 may require in many instances beyond the standard methods of immunofixation agarose-gel electrophoresis and haemolysis (3,31) the determination of Rodgers (Rg) and Chido (Ch) antigens (22,23,24), the identification of rare or aberrant phenotypes in "Western blots" with C4A or C4B specific monoclonal antibodies (6,25,54) and the distinction of C4-alpha A or B chains (47), and C4ß H or L chains (32) by SDS-PAGE for the analysis of haplotypes, silent and aberrant alleles. Monoclonal antibodies recognize two different forms of C4 proteins in the C4A*1/B*3/4 region with low haemolytic activity and C4B epitopes. One has been designated C4BI (54) or C4BHI (6), the other C4"A1" on the basis of its alpha-A-chain property in reduced C4 (6).
Allotypic identification of major bands through treatment of C4 with carboxypeptidase B (50) on one hand and TaqI-C4-RFLP (49) on the other hand are newly introduced methods for the recognition of haplotype constellations in C4 as shown in a family with five remarkable C4 characteristics: *1.* lack of C4A but apparent duplication of B3/B1 on haplotype b) as deduced from individual II,2 with three haemolytically active C4B

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Table 2 : **Complement polymorphisms**

	Alleles : common	rare	silent	recomb. DNA	RFLP	no. of fragments
CHROMOSOME 1 :						
C4BP	1	1	+	+		
H	2	1	+	+		
CR1 (C3BR)	2	1	+	+		
C81 (α / γ -chain)	2	3	+			
C82 (ß-chain)	1	2	+	+		
C1Q	-	-	(+)	+		
CHROMOSOME 6 :						
C4 α-chain: C4A	4	>9	+	+	BamH I	2
C4B	3	>18	+	+	Bgl II	2
C4ß-chain	2	+			Kpn I	4
					Taq I	4
					Xba I/BamH I	3
					Nlq IV	A/B genes
					Eco0 109	A/B genes
C2	1	3	+	+	BamH I	multiple
					Taq I	2
BF	2	>16	+			
F-subtypes	2			+	Taq I	2
S-subtypes (?)	2	2				
CHROMOSOME 11 :						
C1INH	-	-	+	+	HgiA I	?
CHROMOSOME 12 :						
C1R	-	-	+	+		
C1S	-	-	(+)	+		
CHROMOSOME 19 :						
C3	2	>26	+	+	Bgl II	2
					Sst I	3
					Taq I	2
UNASSIGNED :						
C5*)	1	1	+	+		
C6	2	>12	+	+		
C7	1	2	+	+		
D*)	1	1	+	+		
I	2	-	+	+		

*) C5 : only polymorphic in Negroids; D: factor D only polymorphic in Melanesians; C3BR : C3b receptor; LG4 : linkage group 4

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Table 1: **Plasma protein polymorphisms in forensic haemogenetics**

	electrophoresis		isoelectric focusing/ immobilines					
	no. alleles*)	SENF (%)	no. alleles*)	SENF (%)	silent alleles	recomb. DNA	RFLP	chromosomal assignment
HP	2 (>10)	12.9	4 (2 + 10?)	30.6	+	+	+	16
GC(DBP)	2 (>10)	15.5	3 (>36)	29.8	+	+	+	4q
TF	1 (>21)	1	3 (>25)	19.5	+	+		3q
PI	3 (>24)	3.9	5 (>27)	27.0	+	+	+	14
ORM	2	18	3	21	?	+		9
PLG	2 (2)	16.1	2 (>11)	19.8	+	+		?
F13A	2 (3)	13.7	-	-	+	+		6p
F13B	3 (>8)	22.7	3 (>8)	22.7	?			-
A2HS	-	-	2 (5)	17.8	?			3q

*) No. of rare alleles in parenthesis; SENF : single exclusion chance for non-fathers; RFLP : restriction fragment length polymorphism

Most complement components have also been assigned for the chromosomal location of their structural genes by linkage studies, segregation analysis in families or recombinant DNA methods. Two linkage groups, C4 binding protein (C4BP), factor H (ß1H-globulin or H), and C3b receptor (CR1) on chromosome 1 (29,46), as well as C4, BF, and C2 on chromosome 6 (9) have advanced the knowledge on functional similarities and common ancestral genes of the regulatory protein group (C4BP/H/CR1) and the C3 activators (C4/BF/C2).
The formal genetics of the majority of components exhibits one or two common alleles with a limited number of rare variants, such as in C4BP, H, CR1, C81 or C82. In contrast the spectrum of variants has reached a considerable degree in C3, C4, BF, and C6. Polymorphisms of C8, and of C4 (as to be shown later), are complex with two or more structural loci encoding for each of these proteins (34,45).

Inherited complement deficiencies have been described for all complement proteins, many with disease manifestation, some without. Silent or inactive genes are most common in C1INH, leading to hereditary angioedema, where RFLP may now aid diagnosis. Silent genes are also common in C2 and C4, with systemic lupus erythematosus (SLE) or SLE-like diseases in homozygously deficient individuals (26) and many other associated diseases in heterozygous carriers (34,41,43). Furthermore, in individuals with complete C3, C5, C6 or C7 deficiency multiple episodes of infectious complications were seen (cf. also 48).
The structural gene for C3, the first complement polymorphism to be discovered, now widely used in population and forensic genetics, and also first to be located by recombinant DNA, is found on chromosome 19 (18,61). For five components chromosomal locations are still unknown, including factor I, newly described to be genetically determined in a Japanese population (37). C6 and C7 form a separate linkage group (LG4), which remains unassigned in spite of several linkage studies and cloned nucleotides (36).

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Proteins in Plasma and Urine

GENETIC POLYMORPHISMS OF COMPLEMENT COMPONENTS AND OTHER PLASMA PROTEINS

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More than thirty plasma proteins were described to be genetically polymorphic; only a limited number has been applied to forensic haemogenetics on the basis of genetic variability, known mode of inheritance and technological feasibility. The present review will therefore consider, with the exception of immunoglobulins, those plasma proteins which are well established or appear to be most informative. Special emphasis will be placed on new developments in the field of complement genetics.
Nine plasma proteins are commonly used in forensic haemogenetics and have gained attention for their subtypes or were introduced in recent years (Tabel 1). For some of them subtyping by isoelectric focusing or immobiline techniques has resulted in a considerable increase of information as indirectly expressed in the increment of their single exclusion chance for non-fathers (SENF) in paternity expertises, as calculated according to SPIELMANN & KÜHNL (53). This has especially been observed for haptoglobin (HP) (40,56), the vitamin D-binding protein (DBP, formerly GC) (28), transferrin (TF) (12) and alpha-1-antitrypsin (proteinase inhibitor, PI) (2). The regulatory proteins of the coagulation system plasminogen (PLG) (30,51), factor XIIIA (F13A) (53) and its carrier protein factor XIIIB (F13B) (33,53) were more recently applied to forensic haemogenetics. Prospective candidates for future applications are orosomucoid (ORM) (58,63), and alpha-2-HS glycoprotein (A2HS) (55) polymorphisms. Silent alleles were discovered for most of these proteins, recently also for PLG (52), and may lead to erroneous assumption of inverse homozygosity. Besides, partial or total nucleotide sequences were described for the majority of these proteins, but restriction fragment length polymorphisms (RFLP) seen only for HP (42), PI (11), and DBP (13), the BamH I-RFLP being obviously the most useful in the latter.
Chromosomal assignment either from somatic cell hybrids or recombinant DNA has shown the structural genes of TF and A2HS to be located on chromosome 3q, the DBP gene on chromosome 4q (13), the F13A gene distal to HLA-A on chromosome 6 (5,60), and the ORM, PI, and HP genes to be located on chromosomes 9, 14, and 16 respectively (15; for review cf. also 14). Despite repeated attempts the F13B locus is still unknown and localization of the PLG gene must remain doubtful. Two ORM loci were recently identified, ORM1 encoding for the known alleles, ORM2 being monomorphic (63).

Complement components and regulatory proteins of the complement system are outstanding for their extensive genetic heterogeneity, structural polymorphisms having been discovered for nearly all components except C1 and C9, and for most of the regulatory proteins, including the C3b receptor (CR1) (see Table 2). The many complement polymorphisms though exhibit a higher degree of variability only in five proteins with a potential for practical application; these are C3, BF, C4, C6, and C81. All other components possess limited polymorphic features. Factor D and C5, possibly also factor I (C3b inactivator), were found polymorphic only in certain ethnic groups (reviewed in 34,45).

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TABLE 10

DISTRIBUTION OF ALLELES OBSERVED
IN WHITES AND BLACKS

*System	# Alleles Observed	# Alleles 0.001	** New Variants	Population Specific
GC (48)			10	
W	38	35		1C1,1C2,1C6
B	14	9		1A1,1C10,2A3
BF (12)			-	
W	11	7		
B	8	3		F08
F13A (10)			5	
W	10	8		-
B	2	-		-
F13B (12)			7	
W	9	5		4
B	8	3		6
HP (7)			-	
W	7	5		-
B	5	3		2MOD
PLG (14)			4	
W	10	7		M01
B	8	4		B2,M1
TF (17)			-	
W	17	12		-
B	11	6		D1
ACP1 (13)			6	
W	10	7		-
B	8	4		R
PGM1 (27)			-	
W	23	18		-
B	6	2		-

*Genetic marker tested. Number 1 ( ) is total number of variants observed. W = White, B = Black

** Total number or new variants observed.

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TABLE 9

PGM1 GENE FREQUENCIES

	WHITE (No. Tested 38,930)	BLACK (No. Tested 3,492)
1A	0.6355	0.6591
1B	0.1373	0.1393
2A	0.1700	0.1617
2B	0.0565	0.0395
W1	0.00001 (1)	0.0001 (1)
W2	0.00003 (2)	-
W3	0.00010 (3)	-
W4	0.00005 (1)	-
W5	0.00001 (1)	-
W7	0.00004 (2)	-
W9	0.00005	-
W10	0.00004 (3)	-
W11	0.00003 (1)	-
W12	0.00005 (3)	-
W14	0.00003	-
W15	0.00005 (3)	-
W16	0.00001	-
W19	0.00005 (3)	-
W21	0.00003 (1)	-
W22	0.00009 (2)	0.0003 (1)
W26	0.00001	-
W27	0.00004 (1)	-
W30	0.00001 (1)	-

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TABLE 8

ACP1 GENE FREQUENCIES

	WHITE (No. Tested 43,424)	BLACK (No. Tested 3,560)
A	0.35086	0.22458
B	0.59075	0.74551
C	0.05824	0.01180
R	0.00003	0.01713
*A'	0.00006 (1)	0.00014 (1)
D	-	0.00014 (1)
E	-	0.00056 (2)
F	0.00001 (1)	-
*N	-	0.00014 (1)
*P	0.00001 (1)	-
*S	0.00001 (1)	-
*T	0.00002 (2)	-
*U	0.00001 (1)	-

⊕

⊖

F E R B C *T G *P A H I D Tic *N *S A' *U

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TABLE 6

PLG GENE FREQUENCIES

	WHITE (No. Tested 23,754)	BLACK (No. Tested 2,426)
A	0.67888	0.71908
B	0.28414	0.22464
A3	0.01726	0.00412
M1	0.00029	0.03936
M5	0.01177	0.00350
*A4	0.00042 (47)	0.00185
A2	0.00002 (1)	-
A1	0.00072	0.00041
M4	0.00042	0.00021
*M01	0.00352 (65)	0.00082 (1)
*B01	0.00006 (3)	0.00041 (2)
B1	0.00013	-
B2	0.00002	0.00556
*B21	0.00006	-
*QO	0.0035	-

Untreated PLG

⊕

⊖

*A4 A3 A1 A M5 M4 M1 *M01 B *B01 B1 B2 *B21

TABLE 7

TF GENE FREQUENCIES

	WHITE (No. Tested 11,800)	BLACK (No. Tested 1,152)
C1	0.78011	0.86330
C2	0.15381	0.07856
C3	0.05784	0.01432
D1	0.00030	0.03863
C4	0.00055	0.00043
C5	0.00008 (2)	-
C6	0.00047	-
C7	0.00004	-
C8	0.00008	0.00043 (1)
C15	0.00004	0.00073 (1)
B1	0.00004	-
BO-1	0.00008	-
B1-2	0.00131	0.00043
B2	0.00470	0.00217
D2	0.00017	-
D3	0.00004	0.00043
D chi	0.00034	0.00087 (1)

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TABLE 4

F13B GENE FREQUENCIES

	WHITE (No. Tested 22,344)	BLACK (No. Tested 2,380)
1	0.74454	0.34622
2	0.09013	0.56975
3	0.16208	0.06176
4	0.00264	0.00042
6	0.00013	0.01807
*16	0.00013	-
*17	-	0.00084 (1)
*18	-	0.00252 (1)
*19	0.00004 (1)	-
*20	0.00027 (2)	-
*21	0.00004 (1)	-
*22	-	0.00042 (1)

⊕

⊖

*16 *17 1 2 6 *18 *19 4 *20 3 *21 *22

TABLE 5

HP GENE FREQUENCIES

	WHITE (No. Tested 43,310)	BLACK (No. Tested 2,728)
1	0.39651	0.54384
2	0.60254	0.38979
B	0.00002	-
CA	0.00047	0.00434
JO	0.00021 (4)	0.00041
MA	0.00002 (1)	-
2MOD	0.00023	0.06162 (68)

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TABLE 2

BF GENE FREQUENCIES

	WHITE (No. Tested 41,657)	BLACK (No. Tested 3,564)
S	0.80238	0.44725
S07	0.00870	0.00828
F	0.17834	0.51207
F1	0.01024	0.03100
S030	0.00012 (4)	0.00014
S045	0.00010	0.00014 (1)
F025	0.00004 (1)	-
F030	0.00002	-
F055	0.00001	-
F075	-	0.00014 (1)
F080	0.00004	0.00098 (3)
F130	0.00001 (1)	-

TABLE 3

F13A GENE FREQUENCIES

	WHITE (No. Tested 2,152)	BLACK (No. Tested 255)
1	0.7664	0.792
2	0.2314	0.208
3	0.0002	-
4	0.0005 (1)	-
5	0.0002 (1)	-
*6	0.0002 (1)	-
*7	0.0005 (1)	-
*8	0.0002	-
*9	0.0002	-
*10	0.0002	-

⊖

⊕

1 2 3 4 5 *6 *7 *8 *9 *10

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This data gives a more reliable estimate of the frequency of rare and unusual alleles, because of the large populations tested. The data shows that there is a great deal of polymorphism within the protein and enzyme markers routinely used in many laboratories.

TABLE 1

GC GENE FREQUENCIES

	WHITE (No. Tested 36,960)	BLACK (No. Tested 3,330)
1S	0.56503	0.18423
1F	0.15526	0.67222
2	0.27802	0.10706
1A1	0.00004	0.01712
1C10	0.00001	0.01622
1C1	0.00035 (1)	-
1C2	0.00024 (4)	-
1C3	0.00026 (7)	0.00045 (2)
1C6	0.00014 (4)	-
1C12	0.00005 (2)	-
1C13	0.00003 (2)	-
1C16	-	0.00015
1C21	0.00001	-
1C23	-	0.00015
1C25	0.00001 (1)	-
1C31	0.00005 (2)	-
1C32	0.00005 (2)	-
1C33	0.00004 (1)	-
*1C39	0.00001	-
*1C40	0.00001 (1)	-
*1C41	0.00001	-
*1C48	-	0.00015
*1C51	0.00001	-
*1C53	-	0.00015 (3)
2C7	0.00001	-
2C11	0.00001	-
*2C15	0.00001 (1)	-
1A2	0.00003 (1)	-
1A4	0.00001 (1)	-
1A5	-	0.00015
1A7	0.00001 (7)	-
1A8	0.00005 (1)	-
1A10	0.00008 (1)	-
1A11	0.00001 (1)	-
1A12	0.00001 (1)	-
1A19	0.00001	-
*1A25	-	0.00015
1A29	0.00001 (1)	-
2A2	0.00005 (1)	-
2A3	0.00001	0.00135 (3)
2A5	-	0.00045 (1)
2A12	0.00001	-
2A13	0.00001 (1)	-
*2A16	0.00001 (1)	-
*2A17	0.00003	-

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Incidence of Rare Variants Among Serum Proteins and RBC Enzymes in US Whites and Blacks.

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The incidence of rare or uncommon genetic variants, including some population specific markers are difficult to assess due to small sample sizes generally reported. Such markers and the respective gene frequencies are of considerable value in parentage testing and as evidence in criminal investigations. For this study we have accumulated genetic data on the following marker systems markers GC, BF, F13A, F13B, HP, PLG, TF, ACP1, and PGM1 from local white and black populations. Gene frequencies are presented using large sample sizes which provided data on 160 alleles, 32 new variants and 15 population specific markers.

METHODS

Phenotyping of genetic polymorphisms at our institution were performed using techniques previously published by the authors. Isoelectric focusing (IEF) was performed on all genetic marker systems except HP and BF, which were typed by conventional electrophoresis (CE). GC and PGM1 were typed by IEF and CE. Unusual variants and rare variants were in many cases confirmed by the labs of Drs. P. Kuhnl, G. Mauff, H. Cleve, S. Weidinger, J. Constans and Mr. M. Nelson.

RESULTS AND DISCUSSION

Tables 1-9 list the observed gene frequencies of the tested marker systems. The diagrams accompanying these tables are intended to demonstrate the position of the new variants which are marked with an asterisk (*). For a more complete description of the new GC variants contact Dr. J. Constans, Toulouse, France. The number in closed parentheses ( ) to the right of the frequencies lists the number of observed families with this allele. All new variants had family data, but it may not have been listed because it was seen in another population group tested in our lab.

A descriptive summation of the results is not possible in this text, however table 10 gives an overview of the data. Of 160 alleles observed 135 were seen in whites and 70 in blacks. For alleles with frequencies of < 0.001 we observed 104 in whites and 28 in blacks. Sample size may have contributed to this difference. For those alleles seen in both populations we classified them as population specific if they were different by a factor of x10, eg. PLG*M1, ACP1*R. Others,such as GC*1C2, were called population specific if they were seen in only one population on a reoccuring basis.

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in European samples it is possible to estimate European gene frequencies. There are $UMPK^1$: 0,9522 and $UMPK^2$: 0,0478. Because of the expensive staining procedure and the skew distribution UMPK determination should be restricted only to special cases of paterntity testing.

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Table 4: UMPK groups of various ethnical groups

Author	Ethnical Group or Region	No	UMPK[1]	UMPK[2]	UMPK[3]
Table 3	European	2450	0,9522	0,0478	--
GIBLETT et al.(1974)	Native Africa	122	1,000	--	--
	Afro-American	92	0,989	0,011	--
RANZANI et al.(1977)	Italia,Rome,Milan	915	0,9716	0,0284	--
HARADA et al.(1975)	Japanese	635	0,9472	0,0528	--
ZARINAH et al.(1984)	India	121	0,942	0,058	--
GIBLETT et al. l.c.	American Oriental	112	0,929	0,071	--
ZARINAH et al.l.c.	Chinese	125	0,880	0,060	0,060
GALLANCO and	Mestizo	442	0,979	0,020	0,001
SUINAGA (1978)	Warao Indian	64	0,914	0,020	0,086
PETERSEN et al. (1985)	Eskimo,Alaska	92	0,880	0,060	0,060
GIBLETT et al.l.c.	Cree Indian	91	0,868	0,028	0,104
SCOTT and WRIGHT	Athabaskans,Alaska	213	0,862	0,007	0,131
(1978)	Yupik, Alaska	296	0,815	0,052	0,133
	Tlingits, Alaska	55	0,809	0,027	0,164
	Jnupiat, Alaska	228	0,778	0,022	0,200
	Aleuts ,Alaska	56	0,688	0,080	0,232

The chance of exclusion for nonfathers is with 4,3% higher than that of regularly or often determined systems like ADA, AK Ch_1, Kell, Lewis, Lutheran, 6-GPD and Tf. However because of the expensive staining procedure and the skew distribution of the phenotypes in paternity opinions determination of UMPK should be restricted to special cases.

Summary

Uridine-5-monophosphate kinase (UMPK) groups were determined by starch gel electrophoresis according to GIBLETT et al. (1974) and MARTIN (1982) in a random sample of 1003 blood donors and 45 families with 104 children from Schleswig-Holstein. The results were compared with UMPK determinations of other europide samples and of many different ethnical groups. The estimated gene frequencies are $UMPK^1$: 0,9505 and $UMPK^2$: 0,0495. The rare type UMPK (3) was not observed. Because of the excellent agreement of the UMPK groups

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As it is demonstrated in table 3 the distribution of UMPK groups in our sample does not differ from other European investigations except that of RANZANI et al. (1977) with a disproportionate low frequency of UMPK(2). Surprisingly a Japanese sample of HARADA et al. (1975) has the same distribution of UMPK groups as european samples. Since there are no significant differences between the UMPK groups of the samples in table 3 it is allowed to form a combined "European" sample and to estimate European gene frequencies (Table 3, last column): $UMPK^1$: 0,9522, and $UMPK^2$: 0,478.

Table 3: Comparison of UMPK groups and gene frequencies in European samples.

Author:	GIBLETT et al. (1974) *	KUHN et al. (1975)	DRIESEL et al. (1982)	This Paper (1986)	Total
Region:	America(White)	Western Germany		Nothern Germany	
1 obs.	351	316	648	907	2222
U (exp)	(349,2)	(318,3)	(644,8)	(909,7)	
M 2-1 obs.	33	34	62	93	222
P (exp)	(34,9)	(31,8)	(64,4)	(90,9)	
K 2 obs.	1	1	1	3	6
(exp)	(0,9)	(0,9)	(1,8)	(2,4)	
Total	385	351	711	1003	2450
		Genfrequencies			
$UMPK^1$	0,953	0,949	0,955	0,9505	0,9522
$UMPK^2$	0,047	0,051	0,045	0,0495	0,0478

$X^2 = 0,921 \quad X^2_{(6;0,05)} = 12,592$

* The single phenotype UMPK 3-1 has not regarded.

In the other ethnical groups the gene frequencies of UMPK system differ considerably (table 4). Although the gene frequency estimation in the small samples may have great variance table 4 shows that $UMPK^3$ in europide, negride and asiatic populations does not occur or is extremely rare. Africans have obviously the highest frequency of $UMPK^1$ and Xanthodermals of $UMPK^2$. $UMPK^3$ has a high frequency between 0,9% and 2,3% in erythrodermal populations and Eskimos.

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Frequencies of the red cell uridine-5-monophosphate kinase groups (UMPK),E.C.2.7.4.14 in Schleswig-Holstein

V.Sachs, R. Dörner, U.Markmann

In order to compare the frequencies of red cell UMPK groups in Schleswig-Holstein with the frequencies of samples of other investigators we have determined UMPK groups by starch gel electrophoresis according to GIBLETT et al. (1974) and MARTIN (1982) in a random sample of 1003 blood doners.

Table 1 shows the results. The maximum likelihood(ML) estimation of gene frequencies leads to: $UMPK^1$: 0,9505; and $UMPK^2$: 0,0495. The observed and expected values are in very good agreement.The rare type UMPK(3) was not oberserved. A small investigation of 45 pairs of parents with 104 children (table 2) shows a regular inheritance in accordance with the postulated hypothesis.

Table 1: Distribution of UMPK groups in Schleswig-Holstein

Phenotype	Number obs	(exp)	%	ML Estimation of Gene Frequencies
UMPK 1	907	906,2	90,429	$UMPK^1$: 0,9505
UMPK 2-1	93	94,4	9,272	$UMPK^2$: 0,0495
UMPK 2	3	2,4	0,299	
Total	1003	1003,0	100,000	X^2 =0,172 $X^2_{(1;0,05)}$ = 3,841

Table 2: UMPK groups of 45 pairs of parents with 104 children

-Couples*-		-Children UMPK - 1	2-1	2	Total	X^2
UMPK	No	obs(exp)	obs(exp)	obs(exp)		
1x1	24	62 (62)	--	--	62	--
1x2-1	17	12 (17)	22 (17)	--	34	2.940
1x2	1	--	2 (2)	--	2	--
2-1x2-1	3	1 (1,5)	4 (3)	1(1,5)	6	0,667
Total	45	75	28	1	104	3,607

X^2 = 3,607 $X^2_{(3;0,05)}$ = 7,815

* The very rare couples 2-1x2 (0,046%) and 2x2 (0,0006%) were not observed.

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The rare variant was present in heterozygous form with the common Gt 1 gene in all carriers of the family. The variant is slowly moving in electrophoresis compared to the Gt 1 bands. As it is shown in the pedigree of the family (Fig. 3), it was found in three generations.

Beside the two common Gt alleles (Gt 1, Gt 2), several rare ones are known (Negro, Rennes, Indiana, Los Angeles, Berne, Chicago I and II). Among them, only the Rennes, the Indiana, and the Berne variants are slowly migrating in electrophoresis compared to Gt 1. Figures 1 and 2 show, that the present variant is clearly different from the rare Berne variant we found earlier (Scherz et al, 1976). The other two variants (Rennes, Indiana) are no more available for direct comparison, but from their level of activity (6-10%, and 0-40% relative to Gt 1 respectively) (Baker et al, 1966, Schapira et al, 1969) it can be assumed, that they are different. We therefore considered our variant as "new", and we suggest to call it Gt Oron according to the place of residence of our proband. We observed an increase of activity of the erythrocyte enzyme during the first year of life of our proband. The adult carriers of the variant again had a slightly higher activity than the child. One can conclude form these activity levels, that the variant does not have clinical importance when it is present in the heterozygous form with the common type (Gt Oron-1). This assumption is supported by the fact, that the boy developed perfectly well with regular milk diet and that non of the adults who carry the variant reported on any problems of milk incompatability. In this respect, the situation looks similar to the one for the Duarte variant.

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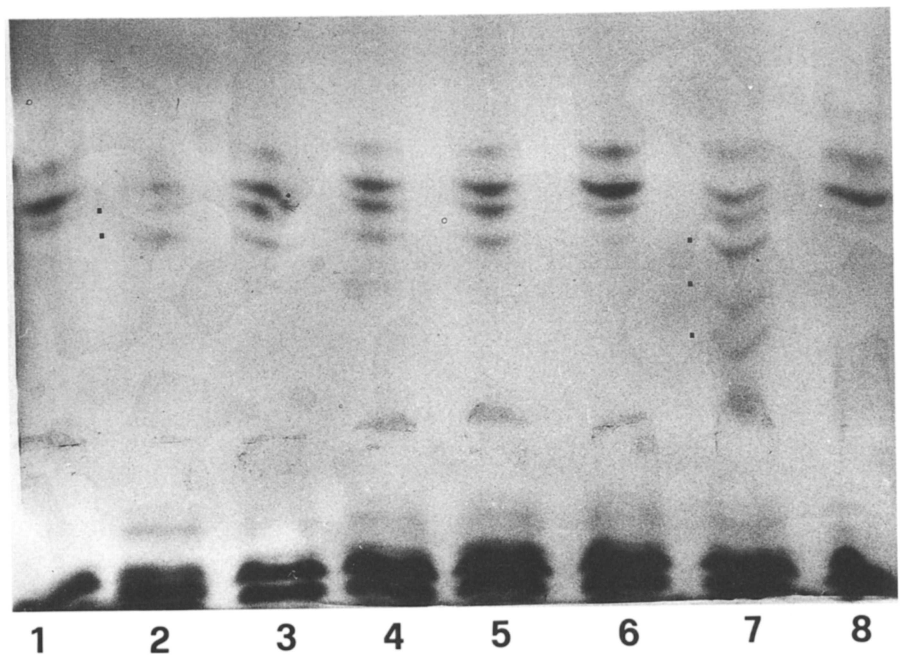
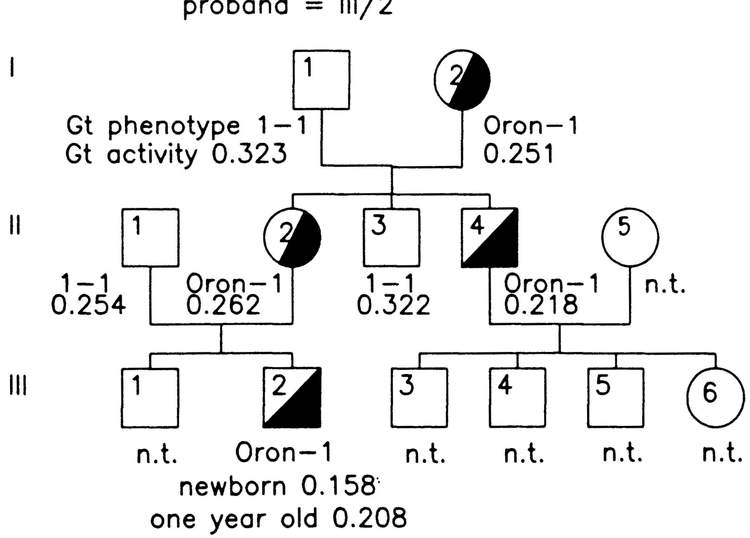


figure 2. isoelectric focusing in agarose gel
Gt 1-1: 1, 6; Gt 2-1: 8; Gt Oron-1: 2, 3, 4, 5;
proband (III/2): 2; Gt Berne-1: 7 anode is at the top

figure 3 Pedigree of family D.
proband = III/2



n.t. = not tested Activity in µMol/Min/gHb

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RESULTS AND DISCUSSION

Phenotype patterns in agarose gel electrophoresis and isoelectric focusing are shown in figures 1 and 2 respecitvely. Enzyme activities are included in table 1.

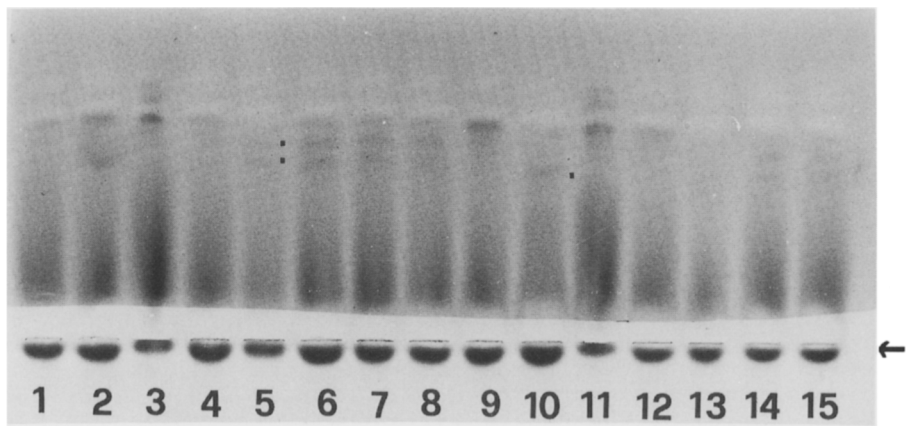
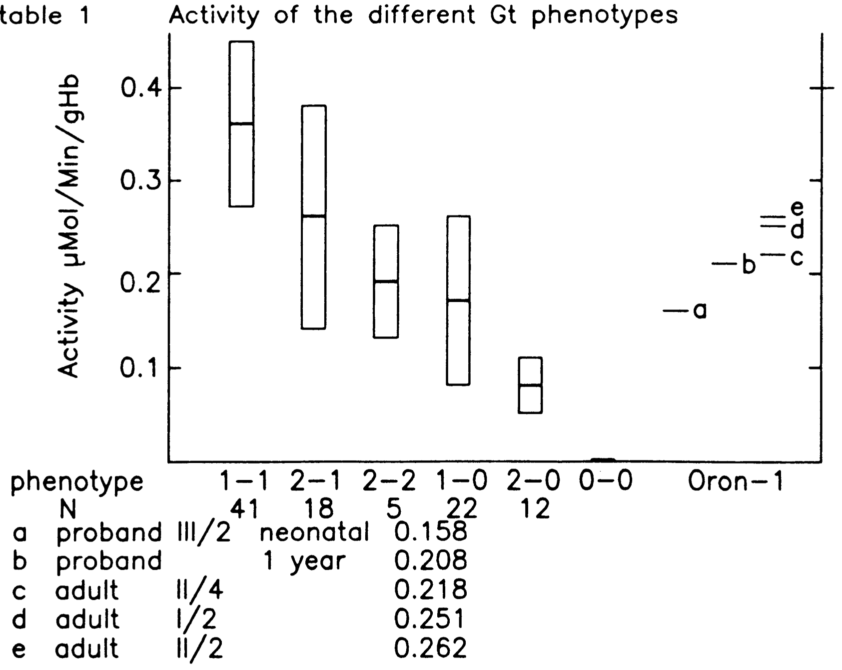


figure 1. agarose gel electorphoresis of galactose-1P-uridyltransferase.
Gt 1-1: 1, 2, 4, 9, 12; Gt 2-1: 3, 11; Gt Oron-1: 5, 6, 7, 8, 13, 14, 15;
proband (III/2): 5, 13; Gt Berne-1: 10
anode is at the top, origin is indicated by arrow

table 1 Activity of the different Gt phenotypes



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A new variant of galactose-1-phosphate-uridyltransferase (Gt Oron)

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INTRODUCTION

The enzyme galactose-1-P-uridyltransferase (Gt), which exhibits a genetic polymorphism, has an important function in the galactose metabolism. Its clinical importance is due to the fact, that the different gene products have different specific enzyme activities, which leads in the case of homozygosity for the null allele to galactosemia (Gt 0-0). Other forms with reduced activities, like the heterozygosity of the Duarte-variant with the null allele (Gt 2-0) are clinically significant as well (Schwarz et al, 1982). On the other hand, the Gt polymorphism has found routine application in forensic hemogenetics.

All newborns in Switzerland are routinely screened for galactosemia by analysis of dried capillary blood spotted on filter paper (Guthrie test) at day four of their life. The proband on whom we report here, has been identified in this screening. His Gt activity was markedly reduced.

MATERIAL AND METHODS

Newborn screening: Guthrie test samples were analyzed for Gt activity with the modified Beutler test (Scherz et al, 1972), and for free galactose by the semiquantitative method of Weidemann (1971).

Quantitative determination of enzyme activity was carried out by using ^{14}C-labelled gal-1P as a substrate (Stucki, 1982).

Phenotyping of Gt was done by electophoresis in agarose gel (Kühnl et al, 1974) and isoelectric focusing in agarose gel (Stucki, 1982).

Case description: The proband (E.D.), a normal newborn baby, was screened for galactosemia at day 6 of life. Reduced Gt activity was detected. The screening test was repeated at day 17 and at day 22, yielding the same result in both samples. Venous blood was then collected and we proceeded to the quantitative determination of the enzyme activity and phenotyping. A rare variant was detected and a family study was undertaken.

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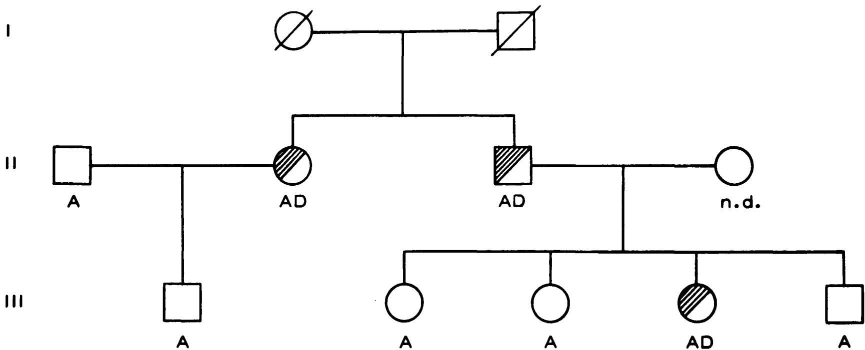
the mother's phenotype. The variant PGD Duisburg, which indicates the father's birthplace, was also found in his sister by paternity testing.



n.d. = not determined

Fig. 3. Pedigree of family Ko. Individuals with the variant PGD Duisburg are indicated with half black symbols

Table 1. Distribution of PGD phenotypes and alleles in a sample from Germany

Phenotypes	Observed n	Observed %	Expected n	Expected %	Allele frequencies
PGD A	1934	95.74	1934.86	95.83	PGD*A = 0.9787
AB	81	4.01	79.08	3.92	PGD*B = 0.0200
B	1	0.25	5.14	0.25	PGD*Var= 0.0013
AR	2				
AH	1				
AD	1				
Total	2020	100.00	2019.08	100.00	

$\sum \chi^2 = 0.0508$; df = 1; $P > 0.20$

The distribution of PGD phenotypes and alleles in a sample from Germany is shown in Table 1. In addition to the two common phenotypes the rare type PGD B as well as the variant phenotypes PGD AR, PGD AH and PGD AD were found in this study. Observed and expected distribution are in good agreement with the Hardy-Weinberg equilibrium. The allele frequencies correspond to those found in other European populations (Prokop and Göhler 1986). Despite the extensive studies carried out on the PGD polymorphism in many different populations, to our knowledge PGD Duisburg is a further rare variant in this system.

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intermediate band of PGD R. The slowest band is an A band, and the middle band is symmetrically placed and has substantially greater intensity than the bands on either side of it. The triplet can be observed only with a high resolution of bands otherwise it looks like a single A band. The banding pattern is not affected by either NADP or dithiothreitol and it is also thermostable. According to its thermostability and electrophoretic pattern this new variant is different to PGD Friendship. Another symmetrically triplet pattern is shown by the faster moving variant phenotype PGD AR and the slower moving PGD AH (Hackney).

Figure 2 gives a diagrammatic representation of the observed PGD phenotypes.

The pedigree of family Ko. is shown in Figure 3. It demonstrates the transmission of the allele PGD*D from the father to one of his four children. Three of these children are homozygote PGD A. There was no possibility to determine

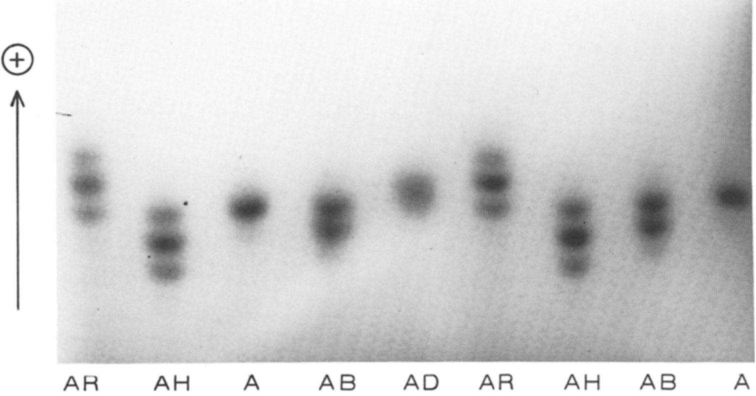

Fig. 1. Banding patterns of PGD phenotypes after starch gel electrophoresis of hemolysates

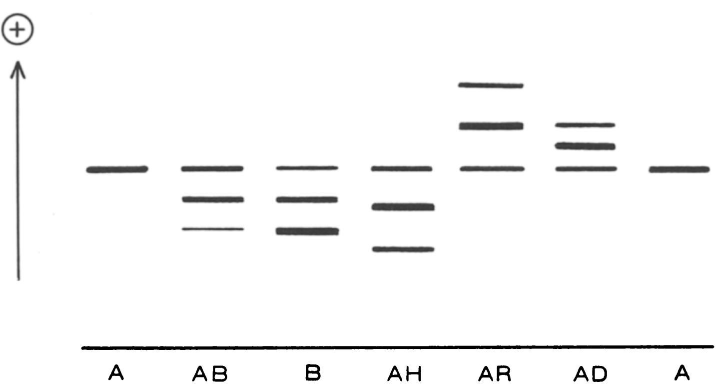

Fig. 2. Schematic representation of the observed PGD phenotypes

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PGD Duisburg: A new Variant of 6-Phosphogluconate Dehydrogenase

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INTRODUCTION

Human 6-phosphogluconate dehydrogenase (PGD-E.C.1.1.1.44) is an important metabolic enzyme in the hexose monophosphate shunt pathway. Genetically determined polymorphism has been described 1963 by Fildes and Parr using starch gel electrophoresis. In addition to the two common phenotypes, PGD A and PGD AB, a number of electrophoretically fast and slow moving rare variants has been observed (Parr 1966; Davidson 1967; Tariverdian et al. 1970; Blake et al. 1974; Spielmann and Kühnl 1982). Inherited quantitative variations of this enzyme have also been reported (Parr and Fitch 1967).

In this paper we describe a new PGD variant along with population data from Germany. This variant which has been tentatively named PGD Duisburg can be differentiated by its electrophoretic mobility and banding intensity. Family studies confirmed its transmission.

MATERIALS and METHODS

The study includes 2020 unrelated individuals from Germany. Hemolysates were prepared by sonification of twice-washed erythrocytes.

Horizontal starch gel electrophoresis was carried out overnight for 15 h at a voltage gradient of 10 V/cm with a 0.1 M phosphate buffer at pH 7.0. NADP was routinely added to the bridge and gel buffer with a final concentration of 2 mg/dl. PGD isozyme bands were visualized on the gel, using the staining agar overlay method described by Harris and Hopkinson (1976).

RESULTS and DISCUSSION

Figure 1 represents the banding patterns of five different PGD phenotypes after starch gel electrophoresis. The newly observed variant PGD Duisburg (PGD D) moves electrophoretically faster than the common PGD A but slower than PGD Richmond (PGD R). Phenotype PGD AD shows a close triplet pattern. The anodal band of this triplet has a similar position as the

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the isozymes is less distinct. This technique therefore represents electrophoresis across a pH gradient or as it has been more specifically termed non-equilibrium focussing. (Divall 1984).

Temperature of coolant and position of sample application: When non-equilibrium focussing occurs both the temperature of the gel and the position of sample application on the gel become important considerations. Both these factors were systematically varied to find the optimum value for both. Moving the origin further from the cathode edge was found to produce incomplete separation of the ADA isozymes. The same effect was observed by decreasing the coolant temperatures below 10°C.

DISCUSSION

This study has shown that the ADA blood group system can be rapidly and reliably phenotyped using isoelectric focussing on ultra thin gels, with a technique which is ostensibly non-equilibrium focussing. Unlike equivalent work with the EsD and PGM systems, this study has not revealed any further ADA phenotypes. However, the technique does offer several advantages over the conventional electrophoretic methods: results can be obtained within 1½ hours of sample preparation and up to 35 samples can be typed on each gel. In our laboratory the IEF technique has been used to group ADA in more than 5,000 samples arriving in a variety of containers and conditions.

Of more interest in considering this technique is the observation that ADA 2 lysates are quite distinct in their isozyme patterns compared to ADA 2.1 lysates. This difference in isozyme patterns appears to remain well defined in lysates kept frozen for several months and this would imply that IEF may offer a more reliable method of phenotyping ADA in bloodstains. Results from preliminary experiments using this technique to phenotype ADA in bloodstains appear to be very encouraging and studies are now being carried out to examine its suitability for this application.

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SOD is itself slightly polymorphic although this aspect of the enzyme was not studied in any way.

ii. Population Studies

The incidence of the three principle ADA phenotypes in three distinct ethnic groups has been studied. Population data and gene frequencies from this study are given in table 1. Gene frequencies in all population groups are in close agreement with previously published data (Steadman 1985).

Table 1. The incidence of ADA phenotypes and gene frequency estimates in three ethnic groups

Ethnic Group	Phenotype	Observed	Expected	χ^2	Gene Frequencies
Caucasian	1	1,829	1,829.943		$ADA^1 = 0.9457$
	2.1	212	210.124		$ADA^2 = 0.0543$
	2	5	5.933		
		2,046	2,046.000	0.164	$= 0.6 < P < 0.7$
Asian	1	398	402.020		$ADA^1 = 0.8581$
	2.1	141	133.006		$ADA^2 = 0.1419$
	2	7	10.974		
		546	546.000	1.960	$= 0.1 < P < 0.2$
Negro	1	170	170.007		$ADA^1 = 0.9913$
	2.1	3	2.975		$ADA^2 = 0.0087$
	2	0	0.017		
		173	173.000	0.001	$= 0.975 < P < 0.95$

iii. Factors Effecting the Isozyme Patterns

While attempting to optimise the conditions for focussing ADA at this pH range, three variables had a noticeable effect on the isozyme patterns observed, these were:- duration of focussing
temperature of coolant
and position of sample application

Duration of focussing: When lysates were focussed for periods exceeding 1 hour, the isozyme patterns travelled further across the gel and became increasingly diffuse. This suggests that the ADA isozymes do not reach their respective pI's after 1 hour's duration but are still travelling to a position of equilibrium on the pH gradient where the separation between

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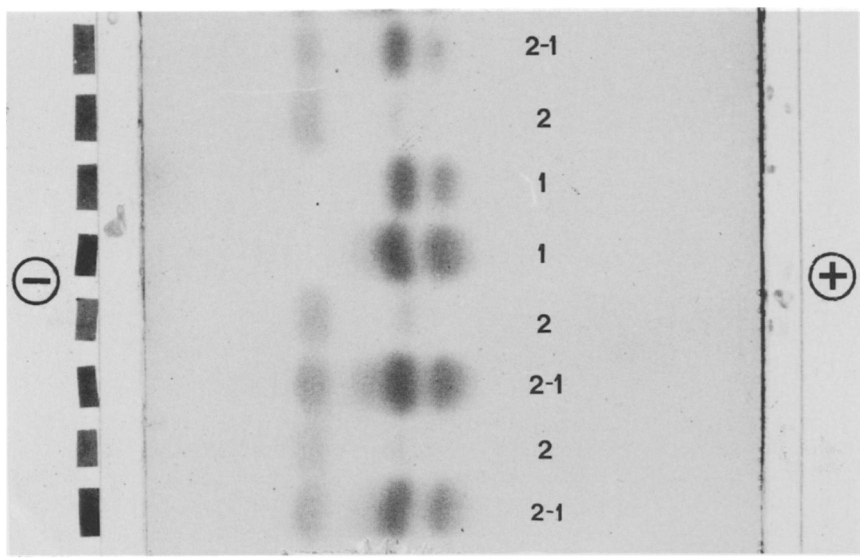

Figure 1 ADA Isozyme patterns of 3 common phenotypes.

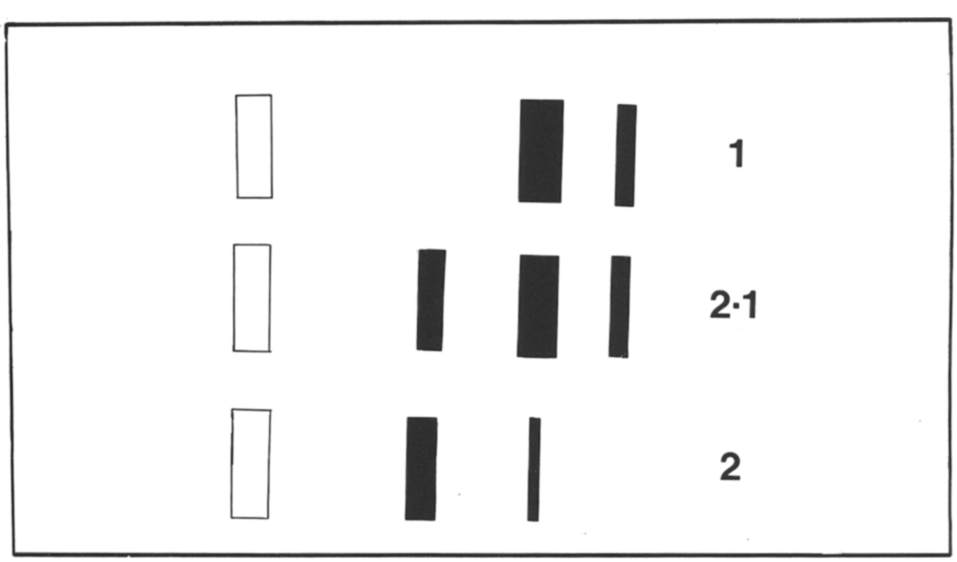

Figure 2 Diagrammatic representation of band patterns.

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Distilled water	40 ml
Acrylogel (BDH Ltd)	1.2 g
"Grade 1" Acrylamide (BDH Ltd)	0.8 g
Sucrose	5 g
Riboflavin 10 mg% sol.	0.4 ml
Ampholine pH 4-6 (LKB)	2 ml

Gels were poured using the flap technique, with top plates treated with a coat of Sigmacote (Sigma). Gels were polymerized using long wave U.V. for 3 hours and subsequently stored at 4°C before use.

Lysates were prepared by adding one drop of 3 x washed packed red blood cells to one drop of .05M dithiothreitol at least one hour prior to application on the gel.

Samples were absorbed onto 4 x 3 mm pieces of filter paper (Whatman No.1) and applied down the long axis of the plate 2 cm from the cathode edge. Anode and Cathode consist of 1 cm Wide Whatman No. 17 filter paper strips soaked in 1M orthophosphoric acid and 1M sodium hydroxide respectively. Gels were focussed for 1 hour at 2000 volts (current and power not limiting) with a coolant temperature of 10°C.

ADA enzyme activity was visualised using an agar based reaction mixture applied to the middle third of the gel.

The following reactants are dissolved in 10 ml of 0.025M phosphate buffer pH 7.5 :

Adenosine (Sigma)	20 mg
Xanthine oxidase (Sigma Grade 1)	0.08 units
Nucleoside phosphorylase (Sigma)	0.8 units
Meldola Blue (50 mg%; Boehringer)	0.2 ml
MTT Tetrazolium (Sigma)	3 mg

This was added to 10 grams of 2% agar and incubated for 30 - 40 minutes at 37°C.

RESULTS

i. Isozyme Patterns

Figure 1 shows a developed plate with the standard 3 phenotypes (the ADA 2 lysate was 2 months old and so weaker). A diagrammatic representation of the band patterns observed is given in figure 2.

With the starch gel electrophoresis technique, distinguishing between ADA 2 and ADA 2.1 is not always straightforward, as the relative strength of the bands can be difficult to assess. With the IEF technique known ADA 2 lysates always gave a very strong first band compared to the faster anodal bands, while ADA 2.1 lysates invariably gave a pattern of banding where the second band was slightly stronger.

In all lysates examined a clear area in an otherwise pale purple background occurs between the 2 band and the most cathodal 1 band. This achromatic region is caused by the enzyme Superoxide Dismutase (SOD: E.C. 1.15.1.1).

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Phenotyping Adenosine Deaminase Using Ultra-Thin Isoelectric Focussing

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INTRODUCTION

Adenosine deaminase (ADA: E.C. 3.5.44.4) catalyzes the conversion of the purine Adenosine to Inosine. Human red cell ADA was first found to be polymorphic by Spencer (Spencer et. al. 1968). Subsequent studies confirmed the existence of two autosomal codominant alleles: ADA^1 and ADA^2, with the ADA^1 allele being much more common than ADA^2 in all ethnic groups. The distribution of allelic frequencies is such that the ADA polymorphism falls into the category of forensic blood grouping systems which are usually not particularly useful but in a certain number of cases provide a strong indicator of identity in criminal and paternity cases.

Most forensic laboratories phenotype ADA using a modification of Spencer's original starch gel method developed by Culliford (Culliford 1978). To date there appears to be no other report of isoelectric focussing as a technique for ADA phenotyping in the literature.

Of all the focussing techniques, ultrathin isoelectric focussing (UTIEF) has proved to be particularly useful when applied to some of the standard polymorphic enzyme systems (Divall 1981, 1983, 1984) and polymorphic serum protein systems (Edwards 1986; Budoule 1987). All methods are fast, reliable and straightforward, with the added advantage of large numbers of samples being tested per plate. More importantly UTIEF offers greatly enhanced sensitivity in the detection of blood group activity in dried bloodstains (Divall 1981, 1983, 1985; Edwards 1986; Budoule 1987).

This paper presents the optimized conditions for IEF on ultrathin gels of the common ADA phenotypes.

MATERIALS AND METHODS

Glass plates measuring 22 x 15 cm were used and a gel thickness of 0.15 mm was achieved by applying PVC insulating tape along the sides of the base plate.

The following solution produces a standard gel composition (5% T, 3% C) for this type of IEF and is sufficient for 10 gels:

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Rare alleles of the PGM1 system could be located between as well as anodal or cathodal to the common PGM1 subtypes, partially overlapping the normally seen major and minor bands of classical subtypes and of PGM2 bands as well. At least 30 different rare variants were found and characterized up to date by conventional electrophoresis and IEF techniques (Dykes et al. 1985). It is necessary to use a minimum of two techniques to completely differentiate rare variants. All currently known variants can be detected by a combination of thin layer agarose gel electrophoresis (AGE), pH 7.4 (as described by Martin 1978) and PAGIF, pH 5-7. Additional IEF in agarose gel (AGIF) at a pH gradient of 4-8 (Dykes 1982) could be useful for identification of rare anodal variants, and eventually PAGIF, pH 6-8, for the identification of rare cathodal variants.

The above mentioned isolated mother-child and child-man exclusions, however, could not be explained - as far as heterozygotes are concerned - by rare alleles or alleles with reduced activity. Mistypings could be excluded according to repeated testing of freshly drawn blood samples and by different experts. Accordingly in some rare cases a clear discrepancy between gene and gene product does exist. It is striking that the PGM1 phenotype 1B,2A(a3-a2), normally occurring in only about 5%, was found in five of the seven cases reported here (Tables 1 and 2). In this connection it may be important, that in a larger Swedish material (n=6800) a significant excess of PGM1 1B,2A(a3-a2) heterozygotes and a corresponding deficit of PGM1 1A,2A(a1-a2) was found (Wetterling 1985). As shown in table 3 Martin (1981) and Kühnl et al. (1977) also observed more 1B,2A(a3-a2) phenotypes than expected, the difference, however, was not significant. In our own material from the Essen area observed and expected numbers of PGM1 1B,2A heterozygotes were exactly identical.

Table 3. Observed and expected numbers of PGM1 1B,2A heterozygotes in different population samples.

	Phenotype PGM1 1B,2A(a3-a2)		
	observed(%)	expected(%)	x^2
Wetterling 1986	349/6800(5.13%)	306.1(4.50%)	6.01
Martin 1981	89/1580(5.63%)	75.6(4.79%)	2.37
Kühnl et al.1977	76/1506(5.05%)	72.89(4.84%)	0.14
Driesel et al.1982	22/496 (4.44%)	24.25(4.89%)	0.22
Bertrams 1987	100/2181(4.56%)	100 (4.56%)	0.00

A corresponding deficit of the phenotype PGM1 1A,2A(a1-a2) existed only in Wetterling's material (x^2=2.52), but was absent in the data obtained by Martin (1981), Kühnl et al. (1977) and Driesel et al. (1982) and in our own analysed material. Thus a deviation from the Hardy-Weinberg equilibrium apparently does not exist.

In conclusion PGM1 subtyping by IEF is complicated by the existence of silent alleles, by at least 30 rare variants, by a number of hyposynthetical variants and last but not least by a number of mysterious, inexplicable isolated mother-child and child-man exclusions. Accordingly we recommend again (Wetterling 1985), not to base an exclusion of paternity on isolated incompatibilities of PGM1 subtypes.

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Silent alleles have been reported by Fiedler and Pettenkofer (1968,1969), Kaplan et al. 1970, Wendt et al (1971), Brinkmann et al. (1973), Horai (1974), Ueno et al. (1976), Schon and Thalhammer (1977), Gahr and Schroter (1981), Herzog and Libich (1982), and Ferrell et al. 1984. The frequency of PGM1*QO probably lies in the order of 0.001.

Partially deficient alleles of the PGM1 system were described by Brinkmann et al. (1972) and by Bertrams et al. (1986). The variant PGM1*W31 observed by Bertrams et al. had a reduced enzyme activity of only 25% of the normal heterozygote PGM1 A1 protein. According to this deficiency the variant could not be detected by conventional electrophoresis on cellulose acetate membranes or agarose gel, even after prolonged staining and overloading of the samples. A similar variant with a reduction to 5% of normal heterozygote activity, described by Brinkmann et al. (1972), could not be identified in conventional starch gel electrophoresis and resulted in an apparent mother-child exclusion in the second order.

It is well known that the PGM1 1B(a3) band in most cases is less intense than the 1A(a1) band. Accordingly PGM1 2-1 heterozygotes often show a characteristic pattern in starch gel or agarose gel electrophoresis with more or less reduced activity of the PGM1 bands ("PGM1 2-1red."). One can expect that "PGM1 2-1red."-types include a high percentage of PGM1 1B subtypes according to the decreased activity of this allotype. In an analysis of altogether 2151 samples with the PGM1 2-1 phenotype Weber found a highly significant association between PGM1 1B(a3) containing phenotypes and samples, which were classified as "PGM1 2-1red." according to faint PGM1 1 bands: PGM1 1B,2A (a3-a2) occurred in 49.71% among 175 "PGM1 2-1red." samples, compared to only 9.92% among 1976 PGM1 2-1 types with normal activity (Figure 2).

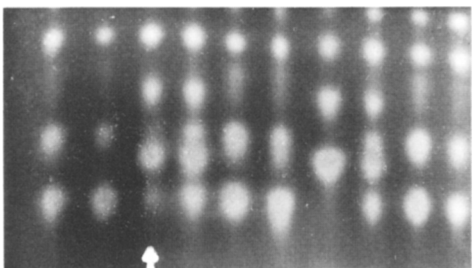

Figure 1. "PGM1 2-1red." phenotypes in starch gel electrophoresis.

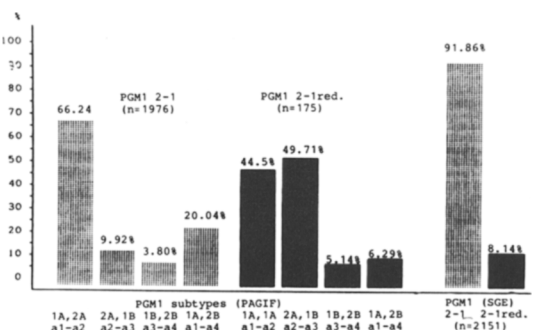

Figure 2. Distribution of PGM1 subtypes among 1976 PGM1 2-1 samples with equal activity of PGM1 1 and 2 bands compared to 175 samples with a reduced PGM1 1 activity ("PGM1 2-1red.").

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Table 2 Isolated father-child incompatibilities in the PGM1 subtype system

	Child	Mother	Putative Father
Weber 1985	PGM1 1A,2A (a1-a2)	1A,2B (a1-a4)	1A (a1)
Weber 1985	PGM1 1A,1B (a1-a3)	1A (a1)	1A,2B (a1-a4)
Höher 1986	PGM1 2A,2B (a2-a4)	1B,2A (a3-a2)	1A,2A (a1-a2)

In the first case the child has inherited the allele PGM1*1A (a1) from its mother and accordingly the allele PGM1*2A (a2) from its father. The accused man was homozygous for PGM1*1A (a1) and could not be excluded by any other investigated genetic polymorphism. The probability of paternity according to Essen-Möller was 99,999999%. Altogether 30 genetic systems have been tested including the HLA antigens. The isolated PGM1 exclusion could be confirmed by repeated blood collections and different investigators. Also the use of additional IEF techniques, like LKB Immobiline Dry Plates, pH 5.6-6.6 (Application note 473) showed the same results.

The second case was also reported by Dr. Weber in 1985. In this case the mother was homozygous for the PGM1*1A (a1) allele and accordingly the child has inherited this allele from its mother and the PGM1*1B (a3) allele from its father. The accused man, however, showed the phenotype PGM1 1A,2B (a1-a4), lacking the allotype 1B (a3). Again a very high probability for paternity was calculated (W=99.9998%). Like in the first case the HLA system was also included.

In the third case, found by Dr. Höher, the child inherited the allele PGM1*2A (a2) from the mother and the PGM1*2B (a4) allele from its father. The disputed father, however, was heterozygous for 1A and 2A (a2-a1). This situation was faced by a probability for paternity of W=99.99%, which resulted from testing altogether 27 polymorphisms including HLA.

In all three cases PGM1 subtyping was performed by PAGIF according to the LKB instruction No 1804-121 with only minor modifications. The characteristics were as follows:

Gel: LKB ampholine PAG plates, pH 5.0-6.5 (T=5.5%; C=3.3%).
Electrode solutions: 0.01 M sodium hydroxide (cathode), 1% acetic acid (anode).
Temperature: 5°C.
Application of hemolysates: ~3µl by sample applications pieces about 2cm from the anode.
Focusing data: Prefocusing for 30min. at 2000V, 15mA and 20W, followed by 150min. focusing time at the same conditions (80min. with and 70min. without application pieces).
Identification of phenotypes by functional agarose overlay according to Sutton and Burgess (1978).

As mentioned above the usefulness of PGM1 subtyping by IEF for paternity testing is complicated by the existence of silent alleles with a relatively high frequency, of rare alleles with reduced activity and of a large number of rare variants.

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On the significance of isolated exclusions in the PGM1 subtype system

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PGM1 subtyping by thin layer polyacrylamide gel isoelectric focusing (PAGIF) has become a commonly used part of paternity testing, since the method is simple, highly reproducible and relatively cheap. According to advantageous frequencies of the four common alleles PGM1*1A or a1(0.6356), PGM1*1B or a3(0.1194), PGM1*2A or a2(0.1910), and PGM1*2B or a4(0.0525) the exclusion probability increases from about 15% with conventional PGM1 typing to over 30% with isoelectric focusing techniques (IEF). The practicability of PGM1 subtyping for paternity testing is, however, restricted not only by a relatively high frequency of silent alleles, of partially deficient alleles, and of a large number of variants, but especially by the occurrence of incompatible heterozygous mother-child pairs and isolated exclusions.

Martin was the first to describe such a mother-child exclusion in 1981 (Table 1). Wetterling (1986) found two more cases of incompatibility among 8686 mother-child combinations and reported an additional case, communicated to her by V. Johnsson/Helsinki. All pairs were typed several times and confirmed by at least two experts. The mysterious inheritance of PGM1 subtypes, as given by IEF,prompted the Scandinavian colleges, not to base any paternity exclusion on results obtained only by PGM1 subtyping (Wetterling 1986).

Table 1 Isolated mother-child incompatibilities in the PGM1 subtype system

	Child	Mother
Martin 1981	PGM1 1A,2B (a1-a4)	1B,2A (a3-a2)
Wetterling 1986	PGM1 1A (a1)	1B,2A (a3-a2)
	PGM1 1B,2A (a3-a2)	1A,2B (a1-a4)
Johnsson 1985	PGM1 1A (a1)	1B,2A (a3-a2)

Our findings of three cases with an isolated exclusion of the putative father according to the results of PGM1 subtyping (Table 2) causes us to point again at the unsolved problem of formal genetics of PGM1 subtypes.

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Table 3. Blood grouping results of the excluded child (C), the mother (M) and the putative father (PF) from the four cases with PGM_1^a exclusion according to the 1st rule of heredity

Blood Group	Case 1			Case 2			Case 3			Case 4		
	M	C	PF	M	C	PF	M	C	PF	M	C	PF
ABO	A_1	A_1	A_1	A_1	A_1	A_2	A	O	A	O	O	O
MNSs	N	N	N	NS	MNSs	MSs	MNSs	MNs	MNs	Ns	MNs	MSs
Rh	cde	cDEe	cDE	CcDEe	cDEe	cDEe	CcDe	cde	CcDe	cDEe	cDEe	cDEe
Kk				k	k					k	k	k
Fy^a				a	a					ab	ab	a
Jk^a				a+	a+							
Xg				a+	a+							
Gm				1,-2	1,-2							
P_1				+	+							
Hp	2	2,1	1	1	2,1	2	2	2	2	2	2	2,1
Gc	2,1S	2,1S	2,1S	2,1S	1S	1S	1S	1S	1S	2,1S	2,1S	2,1S
C3	2	2	2	2	2	2						
Tf	2,1	1		2	2	3,2						
Bf				F	F,S							
Pi				2	2,1							
F13B				2,1	1							
PGM	**a3a2**	**a1**	**a3a1**	**a4a1**	**a3a2**	**a2a1**	**a3a2**	**a1**	**a3a1**	**a4a1**	**a3a2**	**a3a1**
EAP	B	BA	BA	BA	BA	B	CB	CB	B	B	BA	BA
GLO	2	2	2	2,1	1		2	2,1	2,1	1	1	2,1
EsD	2,1	2,1	1	1	1							
AK				1	1					1	1	1
ADA				2,1	2,1					1	1	1
HLA										A2; B12,40	A2,10; B18,40	A9,10; B18,35
Probability of maternity %	95.3			99.9			85.5			98.4		
Probability of paternity %			99.0			89.9			77.7			99.6

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Table 1. Mother-Child exclusions within PGM_1 during 1980-1986 in Sweden (12 682 pairs tested).

Mother	Child a1	a2	a3	a4	a3a2	Total
a1		1	1	2		4
a2	2					2
a3	5					5
a3a2	2					2
a4a1					2	2
Total	9	1	1	2	2	15

Table 2. Distribution of PGM_1^a phenotypes in a sample of 6800 unrelated adult Swedish persons.

Phenotypes PGM_1^a	Observed n	Expected n	χ^2
a1	2804	2751.9	0.9864
a2a1	1186	1241.9	2.5162
a3a1	1313	1356.6	1.3630
a4a1	546	547.6	0.0047
a2	146	140.1	0.2485
a3a2	349	306.1	6.0124
a4a2	127	123.6	0.0935
a3	169	167.2	0.0194
a4a3	132	135.0	0.0667
a4	28	27.2	0.0235
Total	6800		11.3343

For d.f. = 6 $0.10<p<0.05$*
The rare phenotypes are not included.

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Documented mother-child exchange or similar exclusion has not been described in any other blood group system in the Swedish paternity test material.

In some cases there was an incompatibility within the PGM_1 system between father and child, although these fathers had very high indexes (e.g. 976) and accordingly were not excluded as possible fathers.

In other population studies similar maternity exclusions have been observed within the PGM_1 system namely an a3a2-a4a1 exclusion by Martin (1981) and an a3a2-a1 exclusion by Vivian Johnson, Helsinki (personal communication).

It is remarkable that the phenotype a3a2 is present in all these cases. Furthermore, in a large Swedish material (Table 2) a significant excess of the phenotype a3a2 and a deficit of the phenotype a2a1 are observed. When tested for Hardy-Weinberg equilibrium the whole material is very close to the 5 % significance level, mostly due to these deviations.

One explanation for these findings might be that the PGM-locus is relatively sensitive for mutations, which also is indicated by the many described rare alleles.

At the present it is preferable not to base any paternity exclusions on the PGM_1 system only.

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Identification

The PGM phenotypes were visualized by an agar overlay method by Sutton and Burgess (1978), except that 0.08 mM Meldola blue was used instead of phenazine methosulphate.

RESULTS and DISCUSSION

During the years 1980-1986 12 682 mother-child pairs were typed for PGM_1. Fifteen incompatible pairs were found corresponding to a frequency of 0.0012 (Table 1). Four of these were incompatible according to the 1st rule of heredity (opposite heterozygosity) and eleven according to the 2nd rule of heredity (opposite homozygosity). These eleven can consequently be explained by the existence of a silent or an undetected rare allele and are not further discussed here.

The results from the other four mother-child exclusions were verified by new sampling at at least three different occasions with identical and clear results (Fig. 1). The blood grouping results of the mother, the child and the putative father in the four cases are shown in Table 3. Probability values of maternity and paternity were calculated and these values show strong relationship between mother and child. In two families the maternal grandmothers have been investigated and in one family a half-sister without finding any abnormal PGM_1 pattern.

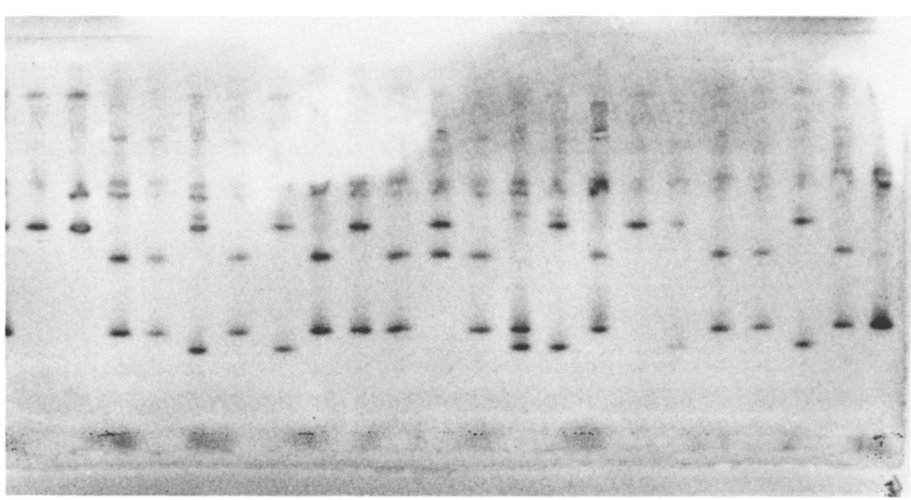

Fig. 1. PGM_1 phenotypes from left to right: a2a1, a2, a2, a4a1, a4a1(GM), a3a2, a4a1(M), a3a2(C), a4a1, a2a1, a4a1(GM), a4a2, a4a1(M), a3a1, a3a2(C), a4a1, a2, a3a2, a4a1(GM), a4a1(M), a3a2(C), a4a1 and a1. GM = grandmother, M = mother and C = child, all from case 4.

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Discrepancy between Gene and Protein Products within the PGM_1 System Shown by Improved Resolution on Immobiline Gels

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INTRODUCTION

Maternity exclusions according to the 1st rule of heredity have been observed within the PGM_1 system (Martin 1981; Wetterling 1986). In order to further investigate this deviation from the genetic rules, four such cases have been retested on LKB immobiline Dry Plates, which so far give the best separation of the PGM_1 isozymes.

MATERIAL and METHODS

Hemolysates

Blood samples from four incompatible mother-child pairs were obtained. Venous blood was collected without additive. Washed and packed red cells were lysed by addition of two volumes redestilled water containing 0.2 % mercaptoethanol as antioxidant. Complete hemolysis was achieved by freezing and thawing.

Isoelectric Focusing

Apparatus: LKB 2217 Multiphor II electrofocusing unit, LKB 2297 Macrodrive 5 power supply, LKB 2219 Multitemp II cooling bath and LKB 2117-915 reswelling cassette for dry gels.
Gel: LKB 1824-560 Immobiline^R Dry Plate pH range 5.6-6.6. Before use the gel is rehydrated in 0.5 % ampholine pH 5-7 over night.
Electrode solutions: 10 mM glutamic acid for the anode and 10 mM sodiumhydroxide for the cathode.
Temperature: +4 °C.
Application: 15 µl of the hemolysate were applied directly on the gel surface 1 cm from the anode (square 12) after prefocusing.
Running conditions: Prefocusing was performed during 30 minutes at 5000 V, 4.0 mA and 12 W followed by 3 hours of focusing at the same adjustment.

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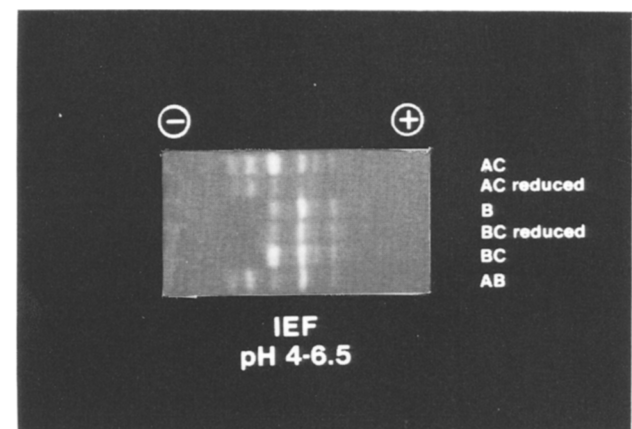
In three families a variant that exhibited a marked decrease in the activity of the ACP1*C gene product was observed on both IEF and conventional electrophoresis. Figure 8 shows a photo of one of the families. The alleged father phenotyped as an ACP1 AC with reduced C activity, the mother as a normal ACP1 B, and the child as ACP1 BC with reduced C activity. Mohrenweiser (1982) describes ACP1*GUA-1, found exclusively in Guaymi Indians, as a variant with low activity migrating in the ACP1 C position on PAGE. He suggests an association of this low activity with an increase in glutathione reductase activity. However, his Indian samples were not run on IEF or compared with our samples from white families. We suspect that they are different quantitative variants.

Fig. 8



CONCLUDING REMARKS

IEF is a fast, easy method for separating ACP1 phenotypes resulting in highly resolved bands. However, many of the rare variants tend to pile up at the cathodal end near the ACP1 A bands. Conventional methods of separation are also needed to confirm the identity of suspected variants.

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ACP1*P was discovered in a white family and one other individual. The bands of this allele appear to have activity that is the reverse of the ACP1 G bands reported by Radam (1982). We also found a family with the ACP1*G allele which confirms that ACP1*P and G are two different variants.

ACP1*T migrates very close to the ACP1 B band on all three methods. This allele was found in two different white families.

ACP1*U, the most cathodal variant on IEF, was discovered in a white family. However, on SGE it migrates between the ACP1 B and C bands.

The allele designated A' was observed in one family and five other individuals, one of whom was black. It migrates in the position of the secondary ACP1 A band on all three techniques. Reversed 'A' activity has been described in the literature by Smerling (1973) and named ACP*K by Turowska (1984). Figures 4-7 are photos of the rare variants on the three methods.

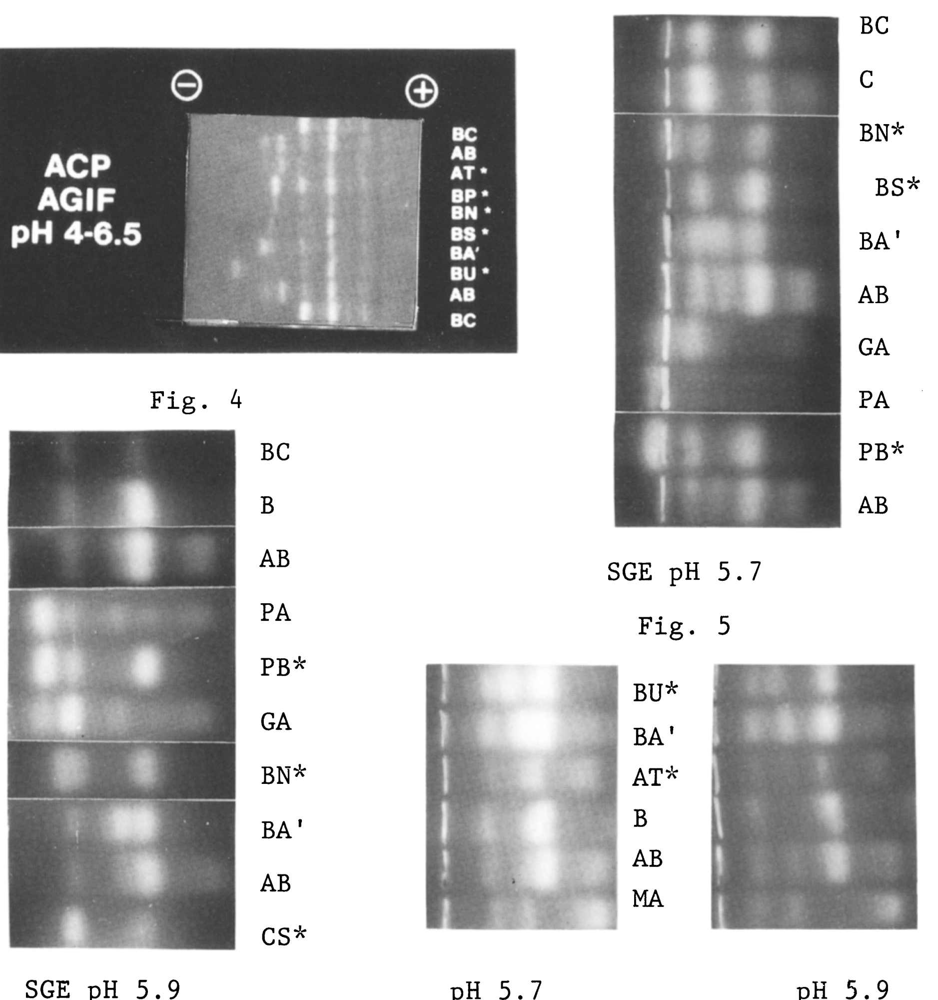

Fig. 4

SGE pH 5.7

Fig. 5

SGE pH 5.9

pH 5.7

pH 5.9

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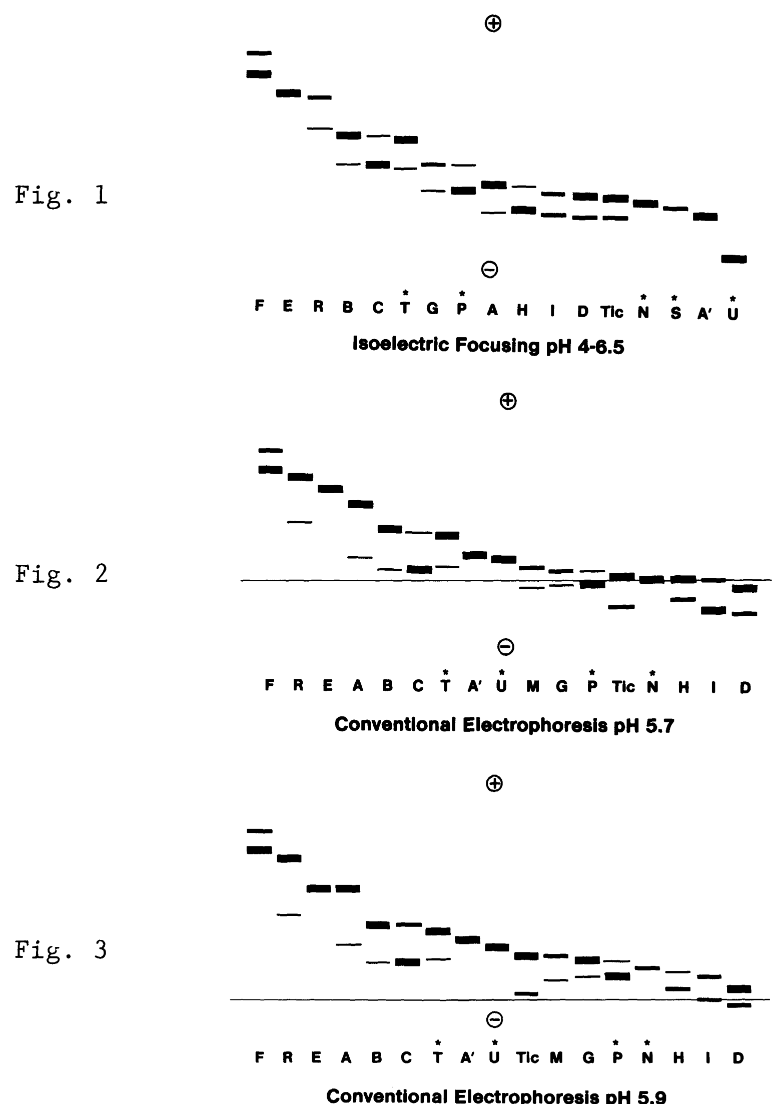

RESULTS AND DISCUSSION

Figure 1-3 show diagrams of the positions of the five new alleles (indicated by *) in comparison to the previously published variants on the three methods of separation. Some of the bands demonstrate shifts in position on IEF. ACP1 M is not distinguishable on IEF from ACP1 A. The new ACP1*S which was found in a white family cannot be distinguished from the ACP1 C band on either conventional method. On IEF, it is seen as a single band migrating just cathodal to the primary ACP1 A band.

ACP1*N, found in a black family migrates just cathodal to the ACP1 C band on SGE but much more cathodal on IEF.

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ACP1 Polymorphism: Five New Variants Detected by Multiple Electrophoretic Methods

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INTRODUCTION

Human red cell acid phosphatase (ACP1, EC 3.1.3.2) is known to be genetically polymorphic. Three codominant autosomal alleles ACP1*A,B and C were first described by Hopkinson (1963) using differences in electrophoretic mobilities. Other variants published to date include ACP1*D, E, F, G. H, I, K, M, R, GUA-1 and TIC-1. This report describes five new variants identified by both isoelectric focusing and conventional electrophoresis. The new alleles show genetic transmission from family data and are named ACP1*N, P, S, T, and U. Further evidence of an allele with reversed ACP1 A band intensity and a variant with reduced ACP1 C activity are discussed.

METHODS

Variant samples were obtained from paternity cases tested in our laboratory. Blood was collected in ACD anticoagulated tubes. The cells were washed three times with saline and hemolysed with an equal volume of deionized water and stored at -20°C until tested.

The ACP1 phenotypes were determined by agarose isoelectric focusing (IEF) on 0.5 mm thick gels containing 0.225 g agarose IEF (Pharmacia) 2.7 g sucrose, 20 ml deionized water and 1.4 ml ampholyte pH 4-6.5 (LKB). Anode and cathode strips contained 1M H_3PO_4 and 0.2 M NaOH respectively. The hemolysate samples were treated with 0.1% 2-ME for 30 minutes and applied 1 cm from the cathode using 3 x 5 mm filter paper wicks. Focusing was performed at 2000 V, 20 mA and 5W for 10 minutes. Sample wicks were removed and the power increased to 10W for an additional 35 minutes. Variant samples were also identified by conventional starch gel electrophoresis (SGE) at both pH 5.7 and pH 5.9 according to Nelson (1984, 1985). On all three techniques band patterns were developed by placing over the entire gel a piece of filter paper soaked in 4-methylumbelliferyl phosphate in a sodium citrate buffer.

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To investigate the nature of this difference purified Bf- and Bs-isozymes were digested by trypsin (Gracy 1977), which cleaves peptide chains at lysine and arginine residues. A chemical difference between the Bf- and Bs-molecules would then be contained in one or more of the tryptic peptides resulting in one or more unique peptides and a number of peptides common to both molecules.

The tryptic digests were analyzed by reversed phase HPLC (fig. 5). Fifteen major peptide peaks were separated with the Bs digest and 17 with the Bf digest. Twelve peaks were common to both isozymes, whereas three peaks (T2, T6 and T17) were unique to the Bs-isozyme and 5 (T3, T7, T9, T10 and T11) were unique to the Bf-isozyme. One Bs peak, Bs-T16, was consistently at least twice as big as the Bf-T16 peak indicating another peptide difference. This observation of multiple peptide differences between the Bf- and Bs-isozymes indicates a chemical difference more extensive than a charge difference on a single amino acid residue.

It seems unlikely that these differences could arise through simple post-translational modification. Given the constancy in the proportion of the isozymes throughout the lifespan of the red cell (Rogers et al. 1978) the modification mechanism would furthermore have to be regulated such that only a defined proportion of the enzyme molecules are processed.

The molecular differences would be explained postulating that f- and s-isozymes are synthesized as discrete molecules. A mechanism involving two closely linked genes coding for the f- and the s-isozyme, respectively, would account for this, but it implies that the mutations giving rise to the A, B and C allelic variation being duplicated in each pair of linked genes, which seems to be a highly unlikely occurrence. However, a possible mechanism, which has been described for several non polymorphic proteins (Noguchi et al., 1986; Leff et al., 1986), is that the ACP1 alleles each undergo alternative splicing at the RNA level to produce a "f"-RNA and a "s"-RNA messenger (fig. 6).

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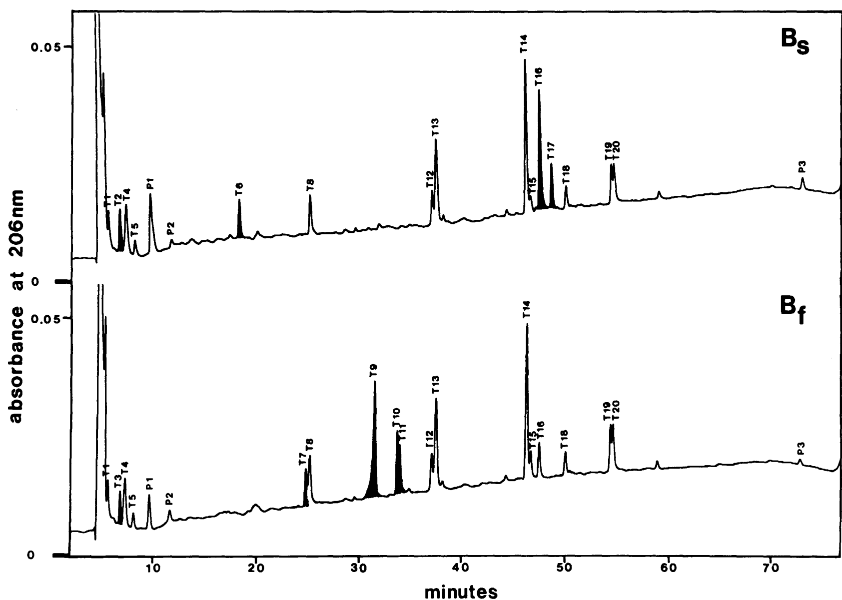

Fig. 5. Reversed phase HPLC analysis of tryptic digests of purified acid phosphatase isozymes of type B using a linear gradient from solvent A (0.1% trifluoroacetic acid in H_2O) to solvent B (0.1% trifluoroacetic acid in 65% acetonitrile (vol/vol). The peptides are numbered T1 through T20. P1, P2 and P3 indicate UV-absorbing peaks contributed by the reagents present in the digestion mixture and by the solvent system.

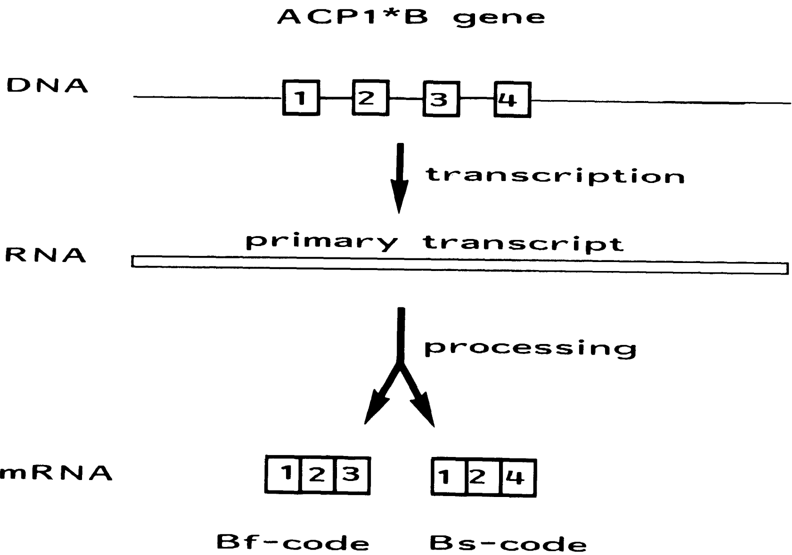

Fig. 6. Hypothetical mechanism generating discrete Bf- and Bs-mRNAs by alternative splicing of the ACP1*B gene.

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and Bs isozymes (Dissing et al. 1979).[1]

If the Bf and Bs isozymes are conformers, unfolding of the molecules should convert them to a common polypeptide chain and refolding would be expected to result in a mixture of the two isozymes (fig. 2). IEF patterns of the native Bf- and Bs-isozymes and of the same isozymes unfolded in 8M urea is shown in fig. 3; it can be seen that the charge difference between the two isozymes persisted under denaturing conditions. Removal of the urea from the denatured isozymes by dialysis resulted in a partial restoration of the enzymatic activity (23% and 17% for the Bf- and Bs-isozyme respectively). Electrophoresis showed that the renatured isozymes had retained their initial mobility -no evidence of interconversion was observed (fig. 4). These findings are clearly incompatible with the conformer hypothesis and they indicate the presence of a chemical difference between the two molecules.

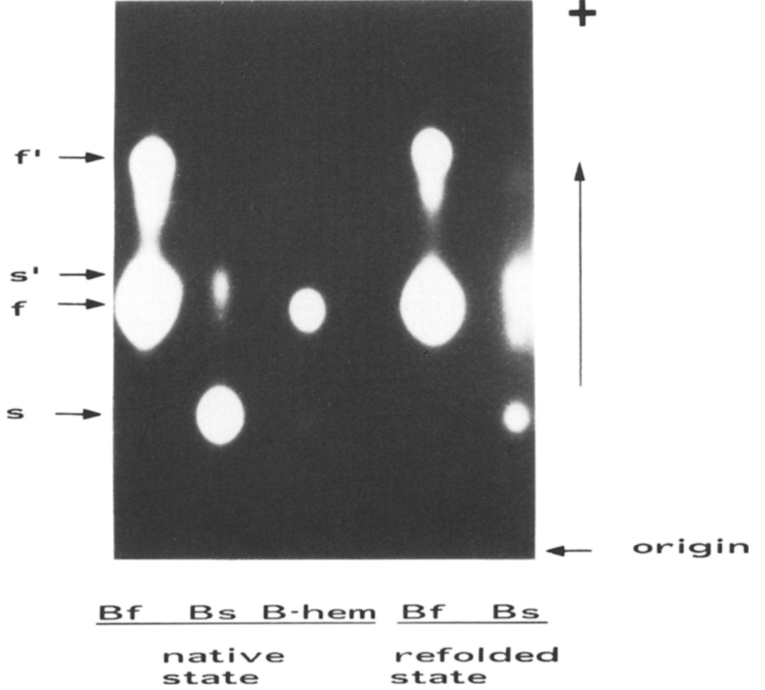

Fig. 4. Starch gel electrophoresis of purified acid phosphatase isozymes of type B in citrate/phosphate buffer, pH 5.9. Lane 1-3 (from left to right): Native isozymes and crude hemolysate (type B). Lane 4 and 5: Isozymes denatured in 8M urea, 10mM dithiothreitol and renatured by removal of the urea. B's and B'f = minor anodal Bs and Bf component respectively.

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[1]A full report on this work will be presented elsewhere (Dissing and Sensabaugh).

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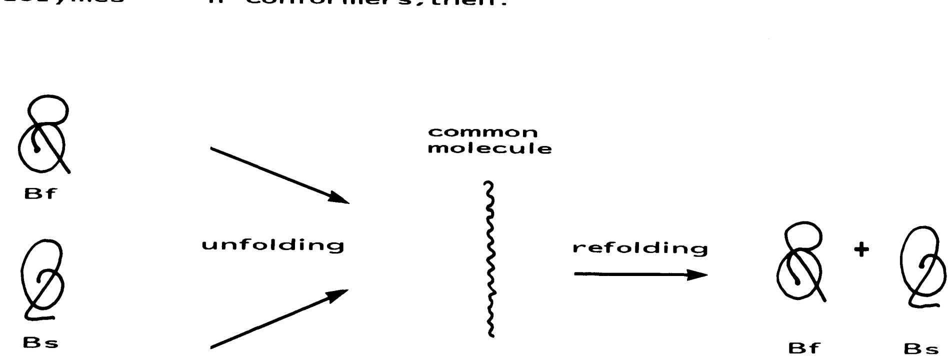

Fig. 2. Considerations about the conformer hypothesis. The example shows the expected behavior of Bf- and Bs-isozymes.

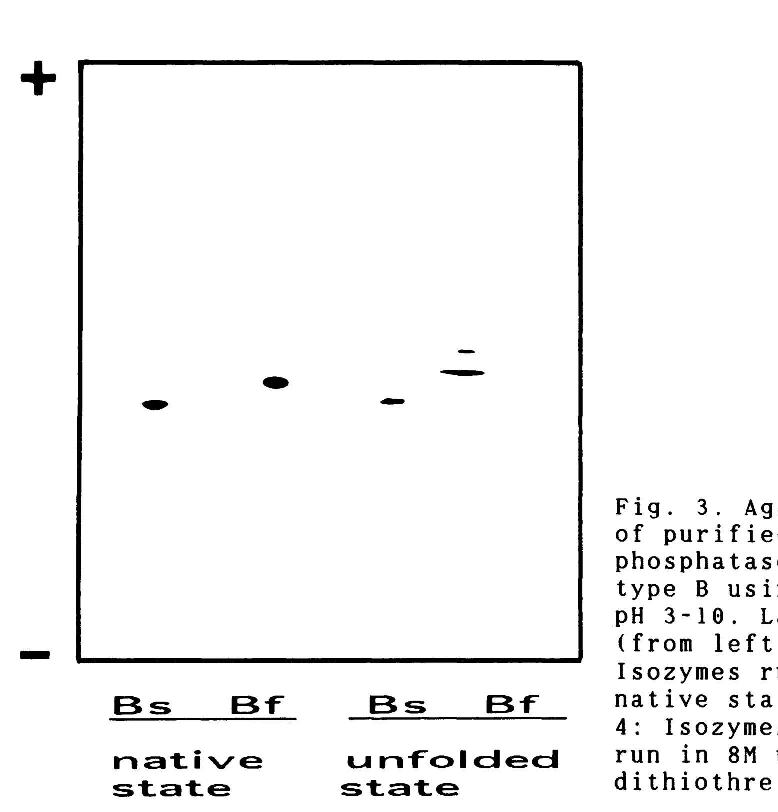

Fig. 3. Agarose gel IEF of purified acid phosphatase isozymes of type B using Ampholine, pH 3-10. Lanes 1 and 2 (from left to right): Isozymes run in the native state. Lanes 3 and 4: Isozymes denatured and run in 8M urea, 10mM dithiothreitol.

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Human Red Cell Acid Phosphatase (ACP1): Evidence for Differences in the Primary Structure of the two Isozymes Expressed by each Allele.

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One of the notable features of human red cell acid phosphatase (ACP1, E.C. 3.1.3.2) is the generation of 2 isozymes (f and s) by each allele. These isozymes differ with respect to electrophoretic mobility (fig. 1) as well as to catalytic, stability and immunochemical properties (Harris 1980, Dissing 1987). The mechanism responsible for this pair wise production of isozymes has long been a puzzle and is of interest not only to the biochemist and geneticist but also to the forensic scientist.

Fisher and Harris (1971) have reported an apparent interconversion of f- and s-isozymes which led to the hypothesis that the isozymes are conformational isomers. Another possibility is that one isozyme is a post genetic modification product of the other, and a third that f- and s-isozymes are synthesized as discrete molecular entities.

The purpose of the present work was to distinguish between these three mechanisms through an investigation on purified Bf

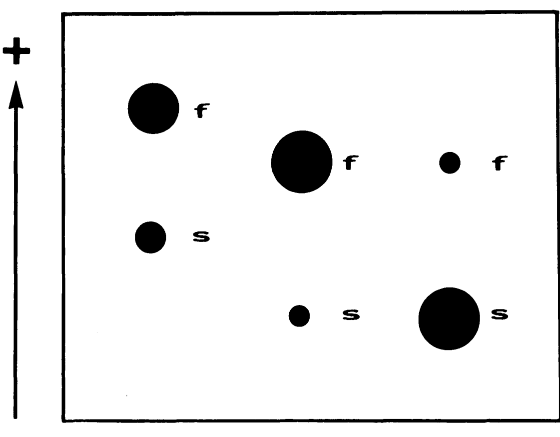

Fig. 1. Diagram of acid phosphatase phenotypes after starch gel electrophoresis in citrate/phosphate buffer, pH 5.9.

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Tab. 6

Blutgruppensysteme

Untersuchungs-Material	Systeme (n)
Erythrozyten-Antigene	17
Serum-/Plasma-Gruppen	32
Blutzell-Lysate	34
Leukozyten-Antigene (HLA) Granulozyten-Marker	2
Gesamtzahl der Systeme	85
Gesamtzahl der Faktoren (> 0,2 %)	ca.1.100
Gesamtzahl möglicher Kombinationen von Blutgruppen-Merkmalen	
a) im HLA-System	$\sim 1{,}7 \times 10^9$
b) übrige Systeme zusammen	$> 2 \times 10^{10}$
Welt-Bevölkerung (1986)	$> 5 \times 10^9$

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Tab. 5

Blutgruppengutachten: Vaterschafts-Ausschluß-Chance (%) durch Untersuchung der Erythrozyten-Antigene, Plasma-Proteine, Isoenzyme und HLA

	Norm-Gutachten	Erweitertes Gutachten	Problemfälle/ Non-Europide
Erythrozyten-Antigene	64,06 %	72,04 %	79,20 %[2)]
Plasma-Proteine	41,96 %	70,64 %[1)]	92,72 %
Isoenzyme	45,63 %	71,78 %	90,85 %
kombiniert	89,64 %	97,68 %	99,86 %
HLA	-	91,00 %	98,30 %
kombiniert	89,64 %	99,80 %	99,998 %

1) einschl. GC-Subtypen, Gm(f), C3 und TF C-Subtypen

2) ohne Dombrock-System

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Tab. 4

II. Isoproteine (Serumgruppen) mit Informations-Gewinn durch IEF bei Europiden

System	Abk.	Jahr	Neue Subtypen (>0.01) - 1986	Neue Varianten (<0,01) - 1986
Alpha-1-Antitrypsin	PI	1975	4	48
Gruppenspezif. Komponente	GC	1977	2	89
Transferrin	TF	1978	3	25
Alpha-2-HS-Glykoprotein	A2HS	1978	2	3
Apolipoprotein E	APO E	1977	3	2
Plasminogen	PLG	1979	2	14
C2-Komplement-Komponente	C2	1976	2	2
C4-Komplement-Komponente	C4 (C4A,B)	1976	10	17
C6-Komplement-Komponente	C6	1978	2	12
C8-Komplement-Komponente	C8 (C81,C82)	1980	6	3
Transcobalamin	TC2	1978	3	6
Gerinnungsfaktor 13B	F13B	1980	3	12
Haptoglobin	HP	1982	4	15
Apolipoprotein A4	APOA4	1982	2	2
Apolipoprotein A1	APOA1	1983	2	-
Alpha-1-B-Glycoprotein	A1BGP	1983	2	-
Total			52	250

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Tab. 3

I. Isoenzyme mit Informationsgewinn durch IEF bei Europiden

System	Abk.	Jahr	Neue Subtypen (>0.01) - 1986	Neue Varianten (<0.01) - 1986
Phosphoglucomutase	PGM1	1976	4	32
Alpha-Fucosidase	FUCA	1975	2	1
Amylase 2	AMY2	1976	2	3
Amylase 1	AMY1	1977	2	4
Esterase D	ESD	1979	3	6
Gerinnungsfaktor 13A	F13A	1979	2	4
Glucose-Dehydrogenase	GDH	1981	3	-
Saure Alpha-Glucosidase	GAA	1982	3	-
Total			21	50

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Tab. 2

Untersuchung verschiedener Isoenzym-Systeme in Blutgruppengutachten

Untersuchungs-Material	Norm-Gutachten	Erweitertes Gutachten	Problemfälle/ Non-Europide
Erythrozyten	ACP1 PGM1 ADA AK PGD ESD GLO GPT	GALT PGP UMPK ALADH SAAH(AHCY)	ESB3 PGM2 GPX SODA G-6PD(X-gekoppelt) PGK (X-gekoppelt)
Leukozyten		FUCA PGM3 MEM (ME2) PEPA	CDA GAA GDH PEPC PEPD GOT2 (GOTM) HK3 FGH
Thrombozyten		F13A	
Plasma		PLG AMY2 CHE1 CHE2	
Speichel		AMY1	
Urin		PG PUM	

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Tab. 1

Entwicklung der Elektrophorese-Techniken (E)

Technik	Jahr	Autoren
Trägerfreie E.	1937	Tiselius
Papier-E.	1952	Kunkel und Tiselius
Stärke-Block-E	1952	Kunkel und Slater
Immun-E. (IE)	1953	Grabar und Williams
Stärke-Gel-E. (SGE)	1955	Smithies
Cellulose-Acetat-Folien-E. (CAF)	1957	Kohn
Polyacrylamid-Gel-E. (PAGE)	1959	Raymond und Weintraub
Agar-Gel-E.	1965	Wieme
Antigen-Antikörper-Kreuz-Immun-E. (CIE)	1965	Laurell
Polyacrylamid-Gradienten-Gel-E. (PGGE)	1968	Margolis und Kenrick
Isoelektrische Fokussierung (IEF) in Dichtegradienten-Säulen	1969	Vesterberg und Svensson
Isotachophorese (ITP)	1970	Haglund
Hochauflösende 2-D-E.	1975	O'Farrell
Polyacrylamid-Gel-Isoelektrofokussierung (PAGIF) ("flat-bed")	1975	Wadström und Smyth; Leaback; Righetti und Drysdale
IEF in immobilisierten pH-Gradienten	1982	Bjellquist et al.

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kindern keine ausreichenden Blutmengen für die Präparation von Leukozytenlysaten (neben HLA-Typisierungen) verfügbar sind. Die aus Literaturangaben erkennbaren Unterschiede der Allelfrequenzen verschiedener Autorengruppen weisen auf technische Probleme bei Trennung und Färbung dieser Isoenzyme hin. Ausschlüsse bedürfen hier im besonderen Maße einer Bestätigung durch andere Labors, um den Beweiswert von Ausschlüssen oder Hinweisen abzusichern.

In den vergangenen 3 Jahren wurden drei Isoenzym-Systeme beschrieben, die allerdings nur eine vergleichsweise geringe Ausschlußchance bieten. Es sind dies die SAHH (EC-3.3.1.1; *2 = 0,023), FGH (EC-3.1.2.12; *2 = 0,11) und ESB3 (EC-3.1.1.1; *2 = 0,035). Vereinfacht wurde die Isoenzym-Diagnostik in jüngster Zeit durch die Entwicklung zeit- und kostensparender Simultan-Typisierungen auf einem Gel. Dabei können folgende Marker-Systeme gleichzeitig abgelesen werden: ADA, AK und PGD; ESD, GLO und CA2; ACP1, ADA und PGM1; ACP1, ESD und GPT.

In Tab. 3 wird der Informationsgewinn bei einigen dieser speziellen Isoenzym-Systeme durch Anwendung der IEF bei Untersuchung von Europiden verdeutlicht. In 8 verschiedenen Systemen wurden insgesamt 21 Subtypen (>0,01) sowie 50 neue Varianten (<0,01) identifiziert. Stellt man den Isoenzymen die Isoproteine (Serumgruppen) gegenüber (Tab. 4), so wird der absolut und relativ wesentlich größere Anteil neuer Subtypen (n = 52) bzw. neuer Varianten (n = 250) bis zum Jahre 1986 evident.

Die hohe Leistungsfähigkeit von Blutgruppengutachten bei der Klärung von Abstammungsfragen spiegelt sich u.a. darin wider, daß in praktisch 100 % aller Fälle Normgutachten angefordert werden, nur noch in etwa 5 % aller Fälle anthropologische Gutachten, bei 1 % sogenannte Tragzeit-Gutachten und <1 % andrologische (Fertilitäts)-Gutachten. Die kombinierte Vaterschaftsausschlußchance durch Untersuchung von Erythrozyten-Antigenen, Plasmaproteinen, Isoenzymen sowie des HLA-Systems geht aus Tab. 5 hervor; sie zeigt den beachtlich hohen Beitrag der Isoenzyme, deren Einbeziehung bei maximalem Untersuchungsumfang einen kombinierten AVACH-Wert von 99,998 % ermöglicht. Insgesamt trägt die Untersuchung von Isoenzymen aus Blutzell-Lysaten sowie teilweise aus Serum oder Plasma wesentlich zur überwältigenden Vielfalt aller möglichen Kombinationen von Blutgruppenmerkmalen bei (Tab. 6). Legt man eine Gesamtzahl von 85 Systemen mit ca. 1.100 Faktoren (>0,002) zugrunde, so ergibt sich auch ohne das HLA-System eine theoretische Zahl von $>2 \times 10^{10}$, die die Weltbevölkerung um das Vierfache überschreitet und so in eindrucksvoller Weise die Möglichkeit der Feststellung der 'biochemischen Individualität' durch Blutgruppenanalysen belegt.

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Betrachtet man die historische Entwicklung der Elektrophorese-Techniken in den vergangenen 50 Jahren, so zeigt sich eine Vielfalt von Trägermaterialien und Kombinationen mit immunologischen und histochemischen Nachweisverfahren (Tab. 1). Als besonders informativ hat sich die Technik der isoelektrischen Fokussierung (IEF) erwiesen, die bereits 1969 in Dichtegradienten-Säulen von Vesterberg und Svensson entwickelt wurde, aber erst in der Modifikation der Flachbett-Isofokussierung auf Polyacrylamidgelen (PAGIF) seit dem Jahr 1975 einen entscheidenden Durchbruch erzielte. Die IEF eroberte sich rasch einen festen Platz in weiteren Angwendungsgebieten außerhalb der Blutgruppengenetik (Serologie) und forensischen Medizin (Spurenkunde), wie z.B. in der klinischen Chemie (Analysen von Hämoglobinen, Protease-Inhibitoren), der Humangenetik (Gen-Kartierung, Populations-Studien), der Immunologie (HLA-Antigene, Immunglobuline), der Zoologie und Botanik (Spezies-Differenzierung durch Analyse der Isoenzym- und Isoprotein-Muster) sowie schließlich der Lebensmittel-Analytik, wo die Qualitätskontrolle und Herkunft tierischer und pflanzlicher Produkte eine wesentliche Rolle spielt.

Für die Blutgruppenserologie werden bestimmte Voraussetzungen für die Einführung eines Isoenzyms in die Routinediagnostik gefordert. Hierzu zählen der Nachweis spezifischer Aktivität, die Untersuchung gereinigten Proteins, der Vergleich der Zymogramme in verschiedenen Techniken, die Überprüfung der Bedingungen des Hardy-Weinberg-Gleichgewichts und Familienuntersuchungen. Individuelle Reproduzierbarkeit, Nachweisbarkeit des Polymorphismus bei Kleinkindern, Untersuchungen zur Lagerungsstabilität und der Existenz modifizierender Gene sowie stummer Allele und quantitativer Varianten kommen hinzu. Schließlich verdienen die Gesichtspunkte der Praktikabilität und eines hohen Informationswertes (allgemeine Vaterschaftsausschlußchance = AVACH) bei gesicherter Formalgenetik Beachtung.

Als Grenzen der Anwendung eines für die Routine-Blutgruppenanalytik potentiell geeigneten Isoenzym-Systems sind folgende Faktoren anzusehen: Ungeklärte Formalgenetik bzw. noch unzureichender Umfang von Bevölkerungsstichproben und Familiendaten, insbesondere der Frequenz stummer Gene; 'schiefe' Allelenverteilung oder zu geringer Informationswert bei einer Frequenz des/der seltenen Allele um 0,01; bei IEF-Systemen fehlende Information im Vergleich zu Standard-Elektrophorese-Techniken; Isoenzym-Nachweis nur an Organ-Extrakten post mortem möglich (Leber, Hirn, Gonaden oder an Plazenten, nicht jedoch an Blutzellen).

Unter Berücksichtigung all dieser Gesichtspunkte ergibt sich heute ein großes Spektrum von Isoenzym-Systemen, die in Blutgruppengutachten untersucht werden können (Tab. 2). Als Untersuchungsmaterial dienen Blutzellen oder Plasma, ausnahmsweise auch Speichel und - zumindest theoretisch - auch Urinproben. Bei Normgutachten beschränkt man sich dabei zunächst auf 8 erythrozytäre, d.h. üblicherweise an Hämolysaten feststellbare Isoenzym-Systeme. Bei erweiterten Gutachten, d.h. fehlenden Ausschlüssen oder unzureichenden W-Werten, kommten 5 weitere erythrozytäre Marker, sowie die in Tab. 2 gezeigten sonstigen Substrate in Betracht. Nur in Problemfällen oder bei Einbeziehung von fremdrassischen Personen ist die Einbeziehung der in Rubrik 3 aufgeführten Systeme indiziert. Bei ihnen sind populationsgenetische Daten und Familienuntersuchungen noch relativ gering, da insbesondere bei Untersuchung von Klein-

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Red Cell Enzymes

Isoenzyme

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Die Analyse von Blutgruppen-Polymorphismen des menschlichen Blutes wurde durch den Ausbau verschiedener Elektrophoresetechniken ermöglicht. Dabei wurden verschiedene Entstehungsmöglichkeiten für Enzym-Heterogenitäten erkannt und genauer definiert. Zum einen können separate Gene zu genetisch unabhängigen Isoenzymen führen, wie dies bei der mitochondrialen und cytoplasmatischen Form der Malat-Dehydrogenase der Fall ist. Polymere Isoenzyme, deren Untereinheiten von zwei Genorten gesteuert werden, können zur Ausbildung höhergradiger Heteropolymere führen, wofür die Laktat-Dehydrogenase (LDH) ein gutes Beispiel bietet. Alle Gene können zur Ausbildung von Isoenzymen mit mono-, di- oder tri-merer Molekülstruktur führen, wie dies bei der Phosphoglucomutase (PGM) oder der Esterase D (ESD) der Fall ist. Sind unterschiedlich viele,gleichartige Einheiten vorhanden, so können polymere Isoenzyme, wie bei der Glutamat-Dehydrogenase (GDH) resultieren. Für die Interpretation von Isozymogrammen ist die Kenntnis epigenetischer Modifikationen primärer Isoenzyme bedeutsam, wobei durch Abspaltung von Neuraminsäureresten, SH-Oxydation, Acetylierung u.a. sekundäre oder aus diesen tertiäre Isoenzyme entstehen. Proteolytische Veränderungen der Polypeptidketten eines Enzyms können dieses in die biologisch aktive Form überführen, wofür die Umwandlung von Chymotrypsinogen in Chymotrypsin ein Beispiel bietet. Schließlich sind allosterische Modifikationen möglich, durch die eine Änderung der Quaternärstruktur des Isoenzyms infolge Anlagerung weiterer Substanzen ausgelöst wird.

Für forensisch-serologische Betrachtungen der genetischen Variabilität von Blutgruppenmerkmalen läßt sich so eine vereinfachte Unterteilung in 4 verschiedene Formen von Blutgruppen-Polymorphismen vollziehen. Zum einen ist dies ein'elektrophoretischer'Polymorphismus mit Mobilitäts-Unterschieden, jedoch gleicher Aktivität der häufigen Genprodukte, beispielsweise der Esterase D (ESD) oder Glyoxalase 1 (GLO). "Struktur-Polymorphismen" mit Aktivitäts- und elektrophoretischen Mobilitäts-Unterschieden der häufigen Genprodukte sind offenbar wesentlich seltener, wofür die Allele der Galactose-1-P-Uridyltransferase GALT*Duarte (Aktivität 25 % der Norm) und GALT*Los Angeles (140 % der Norm) zu nennen sind. 'Aktivitäts-Polymorphismen' charakterisieren Isoenzyme, bei denen sich die Verteilungskurven homo- und heterozygoter Individuen von hyposynthetischen und Defekt-Varianten im optischen Test bzw. der Densitometrie überschneiden (z.B.G-6-PDH). Absolut am häufigsten, jedoch bislang am wenigsten erforscht ist die Gruppe der elektrophoretisch und kinetisch 'stummen' Polymorphismen, bei denen Punkt-Mutationen eine unterschiedliche Primärstruktur ohne Ladungs- oder Aktivitätsänderung erzeugen,z.B. durch Austausch neutraler Aminosäuren an einer biologisch und funktionell nicht relevanten Stelle des Enzymmoleküls.

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II. Electrophoretic Polymorphisms

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Table 2. Ranges of serum IgD concentration with families from the Berlin area

parents			children		
	n	n	l	m	h
l x l	6	23	17(23)	5 (0)	1 (0)
l x m	2	6	0 (3)	6 (3)	0 (0)
l x h	0	0	-	-	-
m x m	5	26	2(6,5)	16(13)	8(6,5)
m x h	1	5	1(1,25)	3(2,5)	1(1,25)
h x h	0	9	-	-	-
total	14	60	20	30	10

of the IgD level, too, does not offer a satisfactory explanation in these cases, since more marked changes in serum IgD concentrations in healthy subjects are very rarely observed (Spiegelberg 1977).

SUMMARY

Indications exist for a genetic influence on serum IgD concentration, possibly there is polymorphism. Our own family studies did not provide any evidence of a formal genetic background.

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fashion of inheritance with two alleles (IgD*l, IgD*h) values result which are present in table 1. In this connection IgD m constitutes the heterozygote type. Presupposing a HARDY-WEINBERG-equilibrium, observed and expected values correlate well.

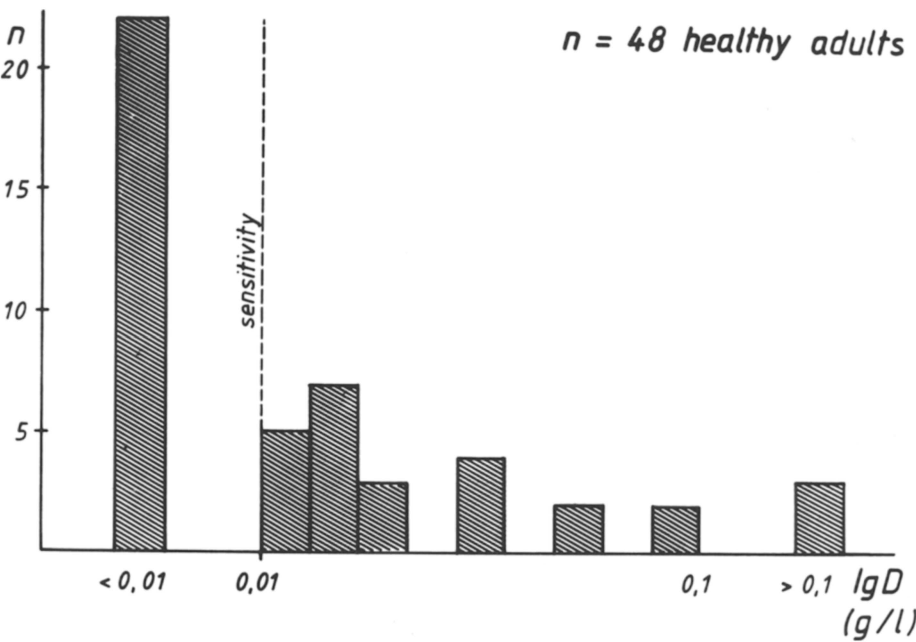

Fig. 1. Serum IgD level of 48 adults measured by means of SRID

Table 1. Ranges of serum IgD concentration: phenotypes with expected values and allelic frequencies in random sample of Berlin population

phenotype	observed (n)	expected	allelic frequencies
l	22	23,52	IgD* l = 0,7
m	23	20,16	IgD* h = 0,3
h	0	4,32	
total	48	48,00	

The result of the family studies is summarized in table 2. With the three parent combinations (l x m, m x m, m x h), distributions in children resulted which correlated well with the expected values for autosomal-codominant inheritance. Concerning the critical combination of IgD l x IgD l, children were found in 5 out of the 6 families who belong to the "medium" or "high" type. This finding cannot be explained by the hypothesis of either a codominant nor that of a recessive-dominant type of inheritance. The assumption of a temporary increase

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Investigations into the inheritance of serum IgD concentration

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INTRODUCTION

Population studies repeatedly showed a trimodal distribution of the serum IgD concentration in adults (Becker et al. 1969; Dunnette et al. 1977; Rowe and Fahey 1965). Consequently, a genetic polymorphism of the serum IgD level was suggested. But also investigations in twins (Allansmith et al. 1969; Lee et al. 1980) and the correlation between IgD concentration in the serum and in the Gm phenotype (Walzer and Kunkel 1974) indicate genetic influences. Family studies (Dunnette et al. 1978) yielded evidence for an autosomal-recessive inheritance of an allele which is responsible low serum IgD concentration.

MATERIALS AND METHODS

IgD levels were measured in unselected sera of 48 healthy Berlin adults by means of single radial immunodiffusion (SRID) - partigen plates and standard of Behring, FRG; conditions of diffusion and intensification of the precipitates with DOPA-solution according to manufacturer's recommendations. Sensitivity 0.01 g of IgD/l. Coefficient of variation between 11.2 % and 18.6 %. All samples were assayed twice.

The same method was used to determine IgD serum concentrations in 14 families of the Berlin area. All children (n = 60) were grown-ups at that time or at least adolescents. No illegitimate children could be detected in the routine typing of the blood-, serum- and enzyme group systems (18 systems) and in the HLA system. All persons were obviously healthy.

RESULTS AND DISCUSSION

The size of the random sample (48 adults) is very small so that it was not possible to clearly determine the mode of distribution (fig. 1). However, the result is not in contrast with data reported by other authors (Becker et al. 1969; Dunnette et al. 1977; Rowe and Fahey 1965) which formed the basis for postulating three IgD concentration ranges: low (l) < 0.01 g/l, medium (m) 0.01-0.1 g/l, high (h) > 0.1 g/l. These ranges may be regarded as phenotypes taking into account the hypothesis of inheritance. Assuming an autosomal-codominant

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Table 4

TYPING FOR Gm, Am AND Km ALLOTYPES BY HAI TEST

1. Ig coated erythrocytes
   - for G1m and G3m typing: 0 R2R2 cells coated with selected anti-Rh antibodies
   - for G2m and A2m typing: 0 cells coated with selected IgG2 or IgA2 myeloma protein by CrCl3

2. Specific anti-Ig allotype antibodies

	advantages	disadvantages
- human antisera	specific	limited, weak
- polyclonal animal antisera	higher titres	pure imm.ag, abs
- mouse monoclonal antibodies	unlimited high titres	labour intensive

3. Reference sera
   - completely allotyped control sera, which discriminate between the allotypes under investigation

4. Test sample
   - serum, plasma, bloodstain or Ig preparation diluted in saline

Table 5

TYPING FOR Ig ALLOTYPES IS VERY USEFUL IN PATERNITY AND BLOODSTAIN ANALYSIS, because

- the system is very polymorphic
- G1m - G2m - G3m - A2m form haplotypes
- Gm and Km are independently inherited, as their genes are located on different chromosomes
- Ig molecules are strong

WRONGLY DRAWN CONCLUSIONS CAN BE PREVENTED by

- determining the right dilution of the antisera
- checking the typing system with reference sera
- testing the samples in more than one dilution
- typing for more than just a few Gm allotypes

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Table 3

FREQUENCIES OF Gm HAPLOTYPES AND A2m GENES IN REPRESENTATIVES OF FIVE CONTINENTS

G1m;G2m;G3m	AFRICA Yorub.	AMERICA Trio	ASIA Taiwan	AUSTRALIA Balimo	EUROPE the Neth
f;n;b	-	-	-	-	0.450
f;..;b	-	-	-	-	0.249
za;..;g	0.007	0.627	0.201	0.091	0.187
zax;..;g	-	0.338	0.043	-	0.098
za;..;b	0.678	-	-	0.164	0.004
za;..;bob1c3c5u	0.203	-	-	-	-
za;..;bob1b4b5c3uv	0.061	-	-	-	-
za;..;bob3b5sv	0.051	-	-	-	-
za;n;b	-	-	-	0.745	-
za;..;bob3b5stv	-	0.035	-	-	-
fa;n;b	-	-	0.755	-	-
others	-	-	0.001	-	0.012
A2m 1	0.174	0.960	0.248	0.646	0.982
A2m 2	0.826	0.040	0.752	0.354	0.018

Fig.1

Pedigree of a family with their Gm–Am phenotypes and deduced genotypes

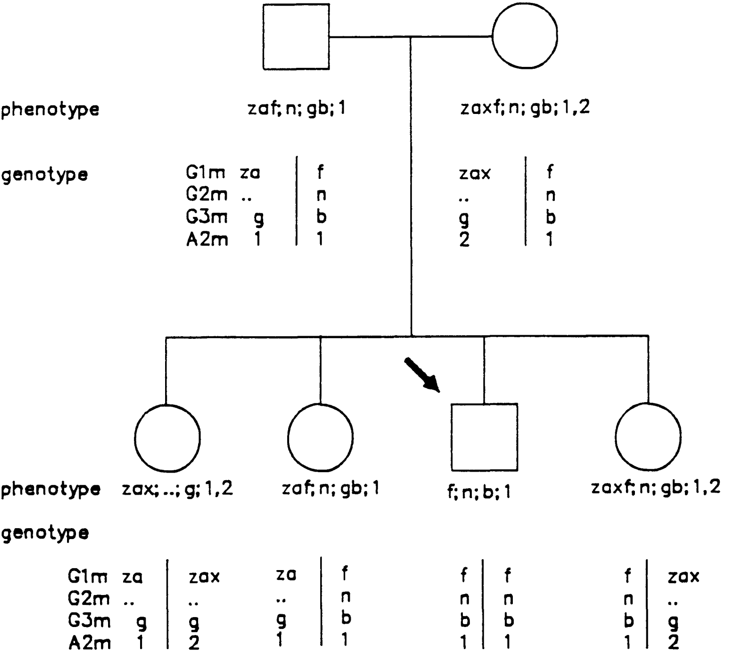

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Table 1

ALLOTYPES OF HUMAN IMMUNOGLOBULINS

Light chains	Heavy chains				
κ	γ1	γ2	γ3	α2	ε
Km	G1m	G2m	G3m	A2m	Em
1	z	n	g1,g5	1	1
2	a		b0,b1	2	
3	x		b3,b4		
	f		b5,		
			s,t		
			c3,c5		
			u,v		

Numerical nomenclature for Gm:

G1m	G2m	G3m		
1=a	23=n	5=b1	14=b4	24=c5
2=x		6=c3	15=s	26=u
3=f		10=b5	16=t	27=v
17=z		11=b0	21=g1	28=g5
		13=b3		

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Wrongly drawn conclusions can be prevented by typing more than just a few Gm allotypes. As, for example, G1m(z) and G1m(f) are mutually exclusive it is very important to type for both, instead of typing for G1m(a) and G1m(f). When G1m(a) and G1m(f) are present it is possible that these allotypes are located on the same molecule.
Another wrongly drawn conclusion can be made after typing of very young children. Children under the age of one year, still possess remnants of maternal IgG, which can inhibit in the assay. In those cases it is advisable to titrate the serumsample, to determine the amount of the different allotypes.

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Table 2 SHORTENED NOTATION OF THE MAIN G3m ALLELES WITH THE DISTRIBUTION OF THE G3m ALLOTYPES ON γ3Fc

G3m alleles	G3m allotypes							
	CH2 domain				CH3 domain			
g	g1	-	-	u	-	-	g5	v
b	-	b1	b4	u	b0	b3	b5	v
c3c5	-	b1	-	u	b0	c3	-	c5
c3	-	b1	b4	u	b0	c3	b5	v
s	-	-	-	s	b0	b3	b5	v
st	-	-	-	st	b0	b3	b5	v

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antibodies. By addition of a serum sample to the anti-allotype antiserum followed by the Ig coated erythrocytes, the agglutination of the antiserum with the coated cells will be inhibited, in cases where the test sample contains the allotype. If the test sample does not contain that particular allotype, the antiserum will agglutinate the erytrocytes (Table 4). The method has been improved by using microtitre plates with V-shape bottom and thin cells suspensions. The sensitivity of the assay could be increased in a way that less test sample and less or weaker antiserum can be used.

Usefulness of Ig allotyping in paternity and other forensic analysis

Because the Gm system is a very polymorphic one, it is very useful in forensic medicine (table 5). The G1m, G2m, G3m and A2m genes are closely located on chromosome 14 and therefore these alleles are inherited as haplotypes. The Km allotypes can be determined with the haemagglutination inhibition method in the same way, but these allotypes are inherited independently of the other allotypes as the κ gene is located on chromosome 2. An advantage for bloodstain analysis is, that immunoglobulins are very strong. Several years old bloodstains can be used for Ig allotyping by dissolving of the stain in saline and using this solution in the HAI assay.

Pitfalls of Ig allotyping

As in most typing systems there are pitfalls to be watched. This can be due to technical errors, but also wrong conclusions can be drawn, due to misinterpretation of the typing results. To prevent the first type of errors it is necessary to check the typing system extensively, with typing reference sera and to determine the right dilution of each antiserum (table 5).

It is also necessary to test the samples in more than one dilution. It is possible that a testserum contains agglutinating antibodies against the Ig coated cells. This has to be checked by incubation of the same dilutions of the testsample with the coated cells. To get rid of these disturbing antibodies the serum can be heated for 10 minutes at 65°C or has to be absorbed with the coated cells before retyping.

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agreed to accept an alphameric as well as a numeric nomenclature (table 1) (ref. 3). A disadvantage of the numeric nomenclature is the extensive and therefore complicated way of expressing the numbers, if there has been typed for more than a few allotypes. Another disadvantage is the fact that for some allotypes two different numbers are still in use for the same allotype, although it has been agreed in 1974 to use the lowest number. More than 10 years later, we still have the situation that the "American" Glm(3) is equal to the "German" Glm(4).

Gm-Am-Em haplotypes

The different heavy chain genes of Ig are linked because they are located close to each other on chromosome 14. Therefore the Glm, G2m, G3m, A2m and Em alleles are inherited in haplotypes, with a low number of crossing-overs within this region. Knowledge of the haplotypes has been achieved by familystudies and extensive population studies. The genotypes, which consists of the two haplotypes, can be deduced from the phenotype by familystudy.

An example of a pedigree of a family with their Gm-Am phenotypes and deduced genotypes is shown in figure 1. For example, it can be deduced that the propositus, marked with an arrow, is homozygous $\underline{G2m^{n}}$, because both parents are G2m(n) positive and his eldest sister is G2m(n) negative. The same way it can be deduced that the eldest daughter, with phenotype zax;..;g;1,2, has two different Glm alleles, namely $\underline{Glm^{za}}$ and $\underline{Glm^{zax}}$.

By population studies it has been shown that some haplotypes are characteristic for a particular race although other haplotypes occur in lower frequencies (Table 3). In Blacks and Orientals Glm(a) acts as an isotypic marker because it is present in all individuals. However in Whites the frequency of Glm(a) positive haplotypes is lower than that of Glm(a) negative ones. On the other hand Glm(f) has never been found in Blacks.

Determination of Ig allotypes

The commonly used method for the detection of Ig allotypes is the haemagglutination inhibition (HAI) test. Human erytrocytes coated with allotype positive Ig are used in this assay with anti-allotype specific

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G3m

The 13 G3m allotypes give rise to a large number of IgG3 alleles, of which the main ones are: $G3m^{g1, g5, u, v}$, $G3m^{b0, b1, b3, b4, b5, u, v}$, $G3m^{b0, b1, c3, c5, u}$, $G3m^{b0, b1, c3, b4, b5, u, v}$, $G3m^{b0, b3, b5, s, v}$ and $G3m^{b0, b3, b5, s, t, v}$. (Table 2)

A shortened notation of these alleles can be used and is called respectively $G3m^{g}$, $G3m^{b}$, $G3m^{c3\ c5}$, $G3m^{c3}$, $G3m^{s}$ and $G3m^{st}$. In Caucasians $G3m^{g}$ and $G3m^{b}$ are the most frequent alleles. $G3m^{c3c5}$, $G3m^{c3}$ and $G3m^{s}$ are typical negroid alleles, although $G3m^{b}$ is frequent in Blacks, too. $G3m^{st}$ is shown to be present in Orientals and in Eskimos.

A2m

The IgA2 allotypes, named A2m(1) and A2m(2), form in practice a two-allelic system encode by the α2 gene. The occurence of the alleles $A2m^{1}$ and $A2m^{2}$ varies a lot between and withing most races (Table 3). As the $A2m^{2}$ allele is rare in Caucasians, the A2m system is not very informative in Whites. In most of the other populations the A2m system is much more informative.

Em

The only IgE allotype discovered, is named Em(1), and has been described by Van Loghem et al in 1984 (ref. 2). Typing for the allotype is not very informative, because it is very common in all races.

Km

Three allotypes of K light chains have been described, named Km(1), Km(2), and Km(3). Before the WHO meeting of 1974, these allotypes were called Inv(1,a,b) or Inv(1,2,3). (ref. 3)

The Km allotypes occur mainly in three alleles: $Km^{1,2}$, Km^{1} and Km^{3}. Km^{3} is the most frequent one in almost all populations (ref. 4). Km^{1} is very rare. Km(1) positive samples almost always are Km(2) positive as well. Therefore Km(2) typing in addition to typing for Km(1) and Km(3) does not give much additional information.

Nomenclature

In 1974 an international exchange of Ig allotype reagents took place followed by a WHO meeting in Rouen, France. At this meeting the members

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Alloantigens on Plasma Proteins

POLYMORPHISMS OF IMMUNOGLOBULINS: Gm, Am and Km TYPING

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Allotypes of immunoglobulins form the most polymorphic genetic system of human serum proteins. Allotypes have been shown on the heavy chains of IgG1, IgG2, IgG3, IgA2 and IgE and on kappa light chains, which are coded by the γ1, γ2, γ3, α2, ε and κ genes respectively. They are called G1m, G2m, G3m, A2m, Em and Km allotypes (Table 1). Most allotypes have originated from the genes by mutation of only one or a few nucleotides resulting in the difference of one or two amino acids. Until now 24 allotypes have been described: four G1m, one G2m, thirteen G3m, two A2m, one Em and three Km allotypes (ref 1).

G1m

The four allotypes that may be present on the γ1 chains of IgG1 molecules are G1m(z), G1m(a), G1m(x) and G1m(f). A maximum of three of these allotypes may be present on the same chain because G1m(z) and G1m(f) are mutually exclusive. Therefore these two allotypes cannot be present on the same chain. The four main combinations of allotypes or so called allelic forms, in which the γ1 gene occurs, are: $G1m^{za}$, $G1m^{zax}$, $G1m^{f}$ and $G1m^{fa}$. The $G1m^{f}$ allele is found in Caucasians only, whereas $G1m^{fa}$ is a typical Oriental allele.

G2m

Because the G2m(n) allotype is up to now the only detectable allotype on γ2 chains, the γ2 gene will occur in the two alleles $G2m^{n}$ and $G2m''$ or $G2m^{n+}$ and $G2m^{n-}$. If a serum is G2m(n) positive, it can be homozygous, that is $G2m^{n}/G2m^{n}$, or heterozygous, that is $G2m^{n}/G2m''$.

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shown in Table 3, the HLA types determined with fibroblasts show a relatively samll number of differences. This slight discrepancy between the two typing, obvserved in 3 antigens among the total number of 32 antigens in A and B locus and 5 antigens among 16 in C locus, is probably due to new expression or disappearance of an antigen during the culture of the fibroblast. In this respect Tada et al. have obvserved that the class II antigens or a new antigens appeared during the culture of T lymphocyte. Thus, a relatively high rate of discrepancy in HLA types in C lous may reflect a loose control of the gene expression. In spite of the above facts, a high rate of concordance of the HLA typing of A and B locus with the use of fibroblasts and lymphocytes showed that our method well fits the practical use. By this method we could determine HLA types of even a 77 years old cadaver using his skin obtained from autopsy at 34 hours after death in Summer; the longest postmortem period, during which fibroblast culture from the skin is possible, remains to be determined.

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Table 2. HLA types determined with cultured fibroblasts and lymphocytes from same living body.

Sample No.	HLA Type Fibroblast	Lymphocyte
1*	A24,26/ Bw61, -/ CW3, W7	A24,26/ Bw61, -/ CW3,W7
2*	A24, -/ B 5, -/ C-, -	A24,31/ Bw52, -/ C -, -
3	A 2,24/ Bw60, 39/ CW2, W7	A 2,24/ Bw60, 39/ CW4,W7
4	A24,11/ BW54, 35/ CW1, W7	A24,11/ Bw54, 35/ CW1, -
5	A24, -/ BW54,W52/ CW1, W3	A24, -/ BW54,W52/ C -, -
6	A24,31/ BW52, -/ CW3, -	A24,31/ BW52,W59/ CW3, -
7	A24,11/ BW54,W61/ CW1, W3	A24,11/ BW54,W61/ CW1, -
8	A24,11/ BW61, 39/ CW7, -	A24,11/ BW61, 39/ CW7, -

* Frozen cells were used.

The results indicated clearly that the HLA types determined with fibroblasts and lymphocytes were essentially the same although a slight difference, probably due to the reasons as will be dis-scussed below, was observed.

HLA Typing of Cadavers

Since the results described above indicated that HLA typing with the use of fibroblast is reliable, we examined 5 cadavers with different age ranging from 0 to 77 and with different postmortem period ranging from 16 to 34 hours. In all of these cases, HLA typing was successful.

Table 3. HLA types of cadavers tested with cultured fibroblast

Sample No.	Hours after death	Age	HLA Type
881	16	0	A1, W33/ B 16, -/ CW3, -
883	18	65	A24, 26/ BW61,W62/ CW3, -
886	18	56	A24, 31/ B 37, 40/ CW2,W3
897	19	17	A24, 26/ B 7, -/ CW7, -
899	34	77	A24,W33/ B 35, 44/ CW3, -

DISCUSSION

In the present report we described that HLA typing of a cadaver could be successfully made by using fibroblasts and slightly modifying the conventional NIH-MCT method for HLA typing of lympocytes. The key point of the modification is the process of pretreatment of fibroblasts for 1 to 2 hours in the culture medium adjusted to pH 8.0; this process keep cells in spherical form, and protects them from sticking to the bottom of tray, and, thus makes it possible to apply directly the premade typing tray, which contains very small amount (1ul) of antisera. As

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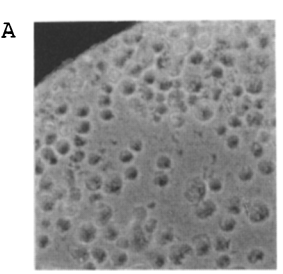
microcytotoxicity test as described under Materials and Methods. Two examples of the wells, which showed a typical positive(A) and negative(B) reactions, are shown by microscopic photographs in Fig.1.

A



B

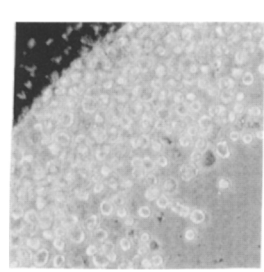


Fig.1 MCT-Positive and -Negative reactions of cultured fibroblast derived from abdominal skin tissue after MCT (A: positive, B:negative).

As shown in above photographs, we can differentiate clearly dead cells and viable cells. Thus according to the percentage of dead cells as a result of the reaction with anti-HLA sera we could score the reactivity as 8(81-100%), 6(41-80%), 4(21-40%), 2(11-20%) and 1(0-10%).

Comparison of HLA Types Determined with Fibroblasts and Lymphocytes

In order to confirm the reliability of HLA typing with fibroblasts,we determined HLA type of fibroblasts and lymphocyte derived from the living body and compared the results. An example of MCT score in positive reactions of cultured fibroblasts and lymphocytes derived from same individual is shown in table 1.

Table 1. MCT positive reactions of fibroblast and lymphocyte from individual No. 1.

		A locus							B locus					C locus									
Specificity	p.c.	A23+24	A23+24	A23+24	A24	A26	A26	A26+W34	BW60+61	BW60+61	BW60+61	BW6	BW6	CW3	CW3	CW3	CW3	CW3	CW3	CW7	CW7	CW7	CW7
Score Fibro.	8	8	8	8	8	8	8	8	8	8	8	6	8	1	1	4	4	6	4	6	1	8	1
Lymph.	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8

The scores of fibroblasts and lymphocytes showed almost complete concordance in A and B locus although the scores of fibroblast in C locus were significantly lower than those of lymphocytes. Thus, by judging the reaction with a score more than 6 as a positive reaction, we determined HLA types of fibroblasts and lymphocytes from 8 control samples (living body)(Table 2).

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at room temperature. After washing few times with fresh medium, the skin was cut into pieces of about 1mm^3 and placed on the bottom of plastic petri dish. The explants were cultured at 37°C in 10% fetal calf serum (FCS), penicillin 100U/ml, streptomycin 100μg/ml. Within 2-10 days after adhesion outgrowth of fibroblasts (or fibroblastlike stromal cells) appeared aroung the "mother explant". When the cell population became large enough to perform subculture (it takes about 1-3 weeks), the cells were detached from the substratum by treating with 0.02% EDTA and 0.25% trypsin (1:1). After washing with DME medium containing 10% FCS, the detached cells were resuspended in the same medium and subcultured.

Preparation of Cells for Use in Cytotoxicity Assays

Cells were detached from a culture plate by treating with EDTA -trypsin and resuspended in RPMI medium (pH 8.0) supplemented with 10% FCS. After allowed to recover for 1-2 hours, the cells were collected by centrifugation and resuspended to give the concentration of 1-2x 10^6 cells/ml by the same medium without FCS and used for cytotoxicity assays.

Cytotoxicity Assays

The original microcytotoxicity test (MCT), which is known to be a conventional NIH method for HLA typing of lymphocytes, was applied also for typing of fibroblast in this study except that the pH of RPMI medium was changed to 8.0 in the latter test. Lymphocyte and fibroblast samples were suspended in RPMI medium pH 7.0 and 8.0, respectively. The suspended cell samples of 1ul (1-2x10^3 cells) were placed in wells of a micro test tray (with 60 wells, Osaka Prefectural Hospital local typing tray), in which 1μl of antisera/well was pre-plated and incubated for 30 minutes at room temperature (about 25°C). Then, 5ul of complement for HLA-A,B,C typing was added to each well. After leaving the samples at room temperature for 1 hours, 2ul of 5% eosin solution was added and after 5 minutes,15ul of 37% formalin was added . After a coverslip was placed on the tray, cells were allowed to settle for several hours, and the trays were read using an inverted-phase microscope.

RESULTS

Reactivity of Fibroblast to Anti-HLA Sera

In order to apply established NIH method for HLA typing of lymphocyte to personal identification of a cadaver by utilizing fibroblasts derived from the abdominal skin of the cadaver, we first tested whether or not fibroblasts, as well as lymphocytes, react with HLA anti-sera. Fibloblast cultures were established from abdominal skin and the cells were subjected to

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Personal Identification by HLA Typing of Cultured Fibroblast Derived from Caderveric Tissues

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INTRODUCTION

It is well known that HLA typing of lymphocyte is extremely useful for personal identification and is often used for paternal testing in the field of legal medicine. Direct application of this method to personal identification of cadavers, however, is sometimes difficult because of relatively low viability of lymphocytes in a cadaver. Other method for HLA typing of a cadaver such as elution method reported by Kashiwade (1986) or absorption method (lymphocyte cytotoxicity inhibition test) reported by Yoshimura (1982) also have some difficulty that, in the case of a cadaver with completely unknown HLA type, these methods require a large amount of highly expensive HLA antibodies, consumes tremendous time and, thus, is not applicable for practical purposes. In an effort to find easy and general application of HLA typing of cadaver tissues, we investigated the possibility of carrying out the test by using conventional typing tray. In this report, we will show that HLA typing of a cadaver can be successfully made by using cultured fibroblast and slightly modifying the conventional NIH method for HLA typing of lymphocytes (Bodmer, 1978).

MATERIALS AND METHODS

Materials

Small amount (about $1 cm^2$) of abdominal skin tissues were obtained by excision at operation of living body, or at autopsy in cadaver to establish fibroblast cell culture. In case of living body, 10-20ml peripheral blood were also obtained before operation to test HLA type of the lymphocyte. Complement used for cytotoxicity assay was purchased from Pel-Freeze Corp.

Establishment and Maintenance of Fibroblast Culture from Tissue

Skin tissue piece obtained was placed in Dulbecco's modified Eagles (DME) medium containing penicillin 500U/ml, streptomycin 500µg/ml and fungizone 20µg/ml, and allowed to leave a few hours

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Case 3.

This case concerns the remains of a male body washed ashore in the beginning of June. The investigations of the pathologists indicated that the remains had been in the sea for 3-6 months. Only most of the sceleton and some tissue from the thighs and the hips remained. The advanced decay allowed determinations of only four genetic marker systems (table 2). Comparisons were with the markers found in two couples of presumed parents neither of whom could have a child of the HLA type of the victim.

Table 2. Serologic Results in Case 3.

	Victim :	0	AcP_1 AB	EsD 1	HLA-A3,A28;B15,B22
Presumed parents:					
1)	Father :	0	AcP_1 B	EsD 1	HLA-A1,A3;B17,B35
	Mother :	B	AcP_1 AB	EsD 1	HLA-A9(24),A11;B15,B35
2)	Father :	A_1	AcP_1 A	EsD 1	HLA-A10(26),A29;B17(w57),B44
	Mother :	0	AcP_1 B	EsD 1	HLA-A1,A11;B5(51),B8

CONCLUDING REMARKS.

In Denmark material is secured at the autopsy for serologic examinations in the case of unidentified bodies or human remains. Determinations of erythrocyte markers are carried out routinely. For HLA determination material may be frozen for investigations when the circumstances of the case makes it necessary.

The examples presented show that serologic examinations may provide an efficient tool for identification, not least when the polymorphic HLA system is included.

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Fig. 1. HLA-DETERMINATION BY MICROABSORPTION AFTER 2-3 WEEKS IN THE SEA.

Examples of Comparison of INITIAL and RESIDUAL ANTIBODY ACTIVITY in Unabsorbed and Absorbed Reagents.

UNABSORBED: 0-0-0-0 DUPLICATE ABSORPTIONS: x-x-x-x & +-+-+-+ TDT: Test Donor HLA-Type

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Case 2.

This case concerns the remains of a female who was found in the harbor of Copenhagen in the end of October after 2-3 weeks in the salt water. The body had been cut into pieces. Seven marker systems could be determined:

Victim : A PGM_1 1F AcP_1 B AK 1 ADA 1 Hp 2 HLA-A28,Aw19*;B21

For the HLA-B locus reduction by absorption of antibody activity was found only in anti-B21 reagents, however, the antigens HLA-Bw46, Bw48,Bw59,Bw67,Bw70,Bw73 were not tested for.
The presence of HLA-A28 could be established through the reduction of antibody activity in anti-A2+A28 sera, but not in anti-A2 sera, (fig. 1). The presence of an HLA-Aw19 factor other than HLA-A29, A30,A31,A32, or Aw33 could be established through lack of reduction of antibody activity by absorption of reagents of narrow anti-Aw19 specificity, f. ex. serum no. R4859 in fig. 1. Besides reduction by absorption of antibody activity directed against some anti-Aw19 components was found in sera of broad specificity: f. ex. the strong anti-A29, but not the weaker anti-A32 activity was absorbed in serum no. 17c, and in serum no. S2415 the opposite was true (fig. 1). No reduction of antibody activity was found by absorption of anti-A10 reagents.
When people of Oriental origin have been typed in the cytotoxic test using the same reagents, a similar reaction pattern has sometimes been observed, indicating that an HLA-Aw19 factor other than HLA-A29,A30,A31,A32 or Aw33 must be present. This HLA-Aw19* antigen could correspond to the one described by Se Jong Kim et al. in a Korean population (1986).
Because of this HLA-Aw19* antigen, and because the HLA type of the victim was most untypical for a Greenland Eskimo, it was concluded that the victim was of Oriental rather than of Eskimoic origin. She was later identified as being a Japanese.

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Case 1.

This case concerns the remains of a woman found in the beginning of September after 2-3 weeks in fresh water lakes. The body had been cut into pieces, and the head and the hands were never found. Serologic examinations gave results for ten marker systems, and the materials used were blood remains, bone marrow and muscle tissue, for HLA determinations only muscle tissue. Comparisons were made with the corresponding marker systems found in the presumed family (table 1). The microabsorption method does not allow the distinction between phenotypic HLA-A2 homozygotes and HLA-A2,A28 heterozygotes, hence both possibilities had to be included for the victim in the statistical evaluations. The presumed parents could have a child of the type HLA-A2,B40//A2,B44, in 1/4 of the cases in which no recombination occurs. The statistical evaluations including all the ten marker systems lead to the result that odds are 1186:1 or 99.92% against 0.08% for the victim being a child of this family against the victim being a random Dane. The results of the serologic examinations provided the final proof for the identity of the victim in this case.

Table 1. The Serologic Results in Case 1.

Victim :	B	Hp 2-1	Gm(a-x-b+)	Inv+	PGM_1 1F-1S	AcP_1 B
	AK 1	6PGD A	ADA 1	HLA-A2;B40,B44 or A2,A28;B40,B44		
Father :	0	Hp 2	Gm(a+x-b+)	Inv-	PGM_1 1F-1S	AcP_1 B
	AK 1	6PGD A	ADA 1	HLA-A11,B35//A2,B40		
Mother :	A_2B	Hp 1	Gm(a+x-b+)	Inv+	PGM_1 2F-1S	AcP_1 BC
	AK 1	6PGD AB	ADA 1	HLA-A11,B7//A2,B44		
Brother :	A_2	Hp 2-1	Gm(a+x-b+)	Inv+	PGM_1 1S	AcP_1 BC
	AK 1	6PGD AB	ADA 1	HLA-A11,B35//A11,B7		
Sister :	B	Hp 2-1	Gm(a+x-b+)	Inv-	PGM_1 1S	AcP_1 BC
	AK 1	6PGD AB	ADA 1	HLA-A11,B35//A2,B44		

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Serology as a Tool for Identification of Dead Bodies.

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INTRODUCTION.

Identification of human remains by the traditional methods of pathology may be impossible or uncertain because of hard damage or decay, and in such cases serologic examinations may provide the necessary evidence for identification. The methods used routinely comprise the ABO blood group antigens, the serumproteins Hp, Gm and Km, the erythrocyte enzymes PGM_1, AcP_1, AK, 6PGD, and ADA. Recently the tissue types, HLA-A,B,C have been added when necessary. While some of these markers degrade soon after the time of death, the HLA antigens have proved remarkably stable, and this rather polymorphic system thus may yield positive evidence for identification.

Methods.

The methods used for blood group, serum protein, and erythrocyte enzyme determinations are all standard techniques as employed for blood stain analysis. The material used is blood remains, bone marrow or muscle tissue.

The microabsorption method used for HLA determination of dead tissue - spleen or muscle - has been described in details elsewhere (Hansen and Gürtler 1981 & 1983). Briefly samples of minced tissue are mixed with 50 µl of HLA antisera selected for the purpose. Absorptions are carried out in duplicate. The recovered serum samples are tested in series of twofold dilutions in the NIH cytotoxic test in order to compare initial and residual antibody activity by means of cells from appropriate testdonors.

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Tabelle 1. Übersicht über die Anzahl von Vaterschaftsausschlüssen bei 89 Ergänzungsbegutachtungen mit 130 Putativvater-Kind-Paaren im Zeitraum 1981 - 1986, in denen die HLA-DR-Merkmale untersucht wurden.

	HLA-A,B,C	HLA-DR	Andere Systeme	Putativvater-Kind-Paare
52 Einmannfälle	+	+	-	7
	+	-	-	3
	-	+	-	1
	-	+	+	1
	-	-	-	40
33 Zweimannfälle	+	+	-	14
	+	-	-	7
	-	+	+	2
	-	+	-	4
	-	-	-	39
4 Dreimannfälle	+	+	-	4
	+	-	-	1
	-	+	-	1
	-	-	-	6

Für die Erstbegutachtung ist eine Untersuchung der DR-Eigenschaften sicherlich nicht zu empfehlen. Als eine der letzten Möglichkeiten zur Klärung strittiger Paternität haben die HLA-DR-Merkmale ihren berechtigten Stellenwert.

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Erfahrungen mit der HLA-DR-Typisierung in der Vaterschaftsserologie

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EINLEITUNG

Berichte über die Anwendung des HLA-Systems in der Paternitätsbegutachtung beziehen sich gewöhnlich auf die Genprodukte der klassischen HLA-A,B,C-Merkmale. Relativ selten sind Beiträge über den Gebrauch von HLA-DR-Antigenen zu verzeichnen. In unserem Untersuchungsmaterial wurde das HLA-System ausschließlich als Ergänzungsgutachten eingesetzt, d.h., daß in der Regel die genetischen Marker ABO, MNSs, Rh, P, K, Hp, Km, Gm, acP, AK, PGM_1, ADA, EsD, GPT, GLO, C3 und Tf vorher untersucht wurden, die zu keinem befriedigenden Ergebnis führten.

Die Einbeziehung des HLA-Systems war angezeigt bei:
1. Einmannfällen mit niedriger Vaterschaftswahrscheinlichkeitsaussage
2. Mehrmannfällen ohne Ausschluß
3. Reinerbigkeitsausschlüssen
4. Defizienzfällen
5. sonstigen Problemfällen.

Die HLA-Typisierungen wurden routinemäßig nach der Zweifarbenfluoreszenz-Methode (van Rood 1975) durchgeführt und nur in speziellen Fällen für die Abstammungsbegutachtung eingesetzt.

ERGEBNISSE UND DISKUSSION

Die Ergebnisse in Tabelle 1 weisen eine weitgehende Übereinstimmung zwischen HLA-DR und HLA-A,B,C bezüglich ihrer Ausschlußquote auf. In 89 Begutachtungen mit insgesamt 130 Putativvater-Kind-Paaren lagen 45 Ausschlüsse und 85 Nichtausschlüsse von der Vaterschaft vor. Isolierte HLA-DR-Auschlüsse wurden in 6 Fällen beobachtet. In 3 Fällen war ein DR-Auschluß mit einem Subtypenausschluß des Gc- bzw. Hp-Systems vergesellschaftet, während bei 11 Putativvater-Kind-Konstellationen alleinige HLA-A,B,C-Ausschlüsse vorlagen.
In Anhängigkeit von der Qualität der Antiseren ist der Beweiswert von HLA-DR-Merkmalen differenziert zu betrachten. Die Merkmale HLA-DR1 bis HLA-DR5 sowie HLA-DR7 gelten als relativ sicher bestimmbar. Problematischer ist die Beurteilung von HLA-DRw6, DRw8, DRw9 und DRw10. Die Bestimmung von DRw10 neben dem gleichzeitigen Vorhandensein von DR1 kann infolge Kreuzreaktivität Schwierigkeiten bereiten. Große Zurückhaltung ist derzeit bei den Splits (DRw11 bis DRw14) geboten. In erweiterten Vaterschaftssachen können DQw-Eigenschaften hilfreich sein.

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cultures, is only present in the Cape Coloureds. This may be explained by the South-East Asian contribution to the Cape Coloured genome. The delta values of HLA haplotypes in strong positive linkage disequilibrium in the Cape Coloureds were approximately half that seen in SA Caucasoids, Xhosa and San, an indication of a hybrid population of recent origin. The genetic distances also indicate that the Cape Coloureds are a hybrid population derived from the SA Caucasoid as well as Southern Africans such as the Xhosa and San.

The HLA frequencies of the **Xhosa** are significantly different from either the Caucasoids or the Cape Coloureds. They have the HLA gene, antigen and haplotype frequencies characteristic of other Southern African Negroes, with evidence of the assimilation of the Khoisan, as seen in the relatively high frequency of Aw43.

In general the **San** resemble the Negroes or even surpass them in many of the features by which Africans generally differ from non-Africans e.g. the very high frequencies of Aw43, Bw58, Cw6 and DRw6. There are, however, also distinct differences in HLA frequencies between these two groups e.g. the raised A3, B8 and Bw41 frequencies and the complete absence of Bw42 in the San.

It has been shown that the HLA system is extremely powerful for the testing of disputed parentage in the varied populations of Southern Africa. Accurate and reliable estimation of an individual population's HLA gene frequencies is a prerequisite for the use of this system in disputed parentage testing, especially when the populations concerned exhibit marked heterogeneity, as is the case in Southern Africa.

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We used two theoretical child-mother-alleged father trios (Table 6) to assess the Paternity Index (PI) and the likelihood of paternity (W) (Essen-Möller 1938; Pohl 1982). A large variation was observed when the frequencies of the four different populations were used in these calculations. In example 1 given in Table 6, we used classic Xhosa phenotypes and in example 2 classic Caucasoid phenotypes. The results clearly illustrate the importance of using the appropriate population frequencies. It is also interesting to note that in the Cape Coloureds, a hybrid population, where the HLA haplotype frequencies are all relatively low, even for the most common haplotypes, the PI and W values never reached the same levels as in the established populations.

These examples stress the necessity of the use of local gene frequencies, based upon reliable data. Bias due to insufficient knowledge of the HLA system and inadequate HLA gene frequencies may have an important effect upon the inclusionary estimates of the system.

Table 6. Two examples of the calculations of the PI and W (%) values in the four Southern African populations reported, using the phenotype frequencies of these populations.

	Example 1		Example 2	
Alleged Father	A1,3	B7,8	A30,28	Bw42,w70
Child	A1,X	B8,Y	A30,X	Bw42,Y
Mother	A24,X	B27,Y	A23,X	B45,Y
Population	**PI**	**W(%)**	**PI**	**W(%)**
SA Caucasoids	7.18	87.78	547.56	99.82
Cape Coloureds	21.80	95.61	27.17	96.45
Xhosa	46.89	97.91	7.46	88.18
San	881.58	99.89	2,176.79	99.95

CONCLUSION

The HLA haplotype and gene frequencies in the **SA Caucasoid** group are essentially the same as in European Caucasoids. However, it is clear that a certain amount of inter-population gene exchange has occurred in Southern Africa as is evident by the presence of the unique Khoisan allele Aw43 in SA Caucasoids.

The **Cape Coloureds** now have HLA frequencies that are unique. In some instances the phenotype frequencies are closer to the Caucasoids e.g. HLA-A11 is present in 12 percent of SA Caucasoids and Cape Coloureds, but only 0.3 percent of the Xhosa. In other instances the frequencies approximate the Negro frequencies e.g. Bw58 which is present in 16 percent of Cape Coloureds, 30 percent of Xhosa and virtually absent from SA Caucasoids. It should be noted that Bw48, said to be characteristic of early Mongoloid

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laboratory, DR2s and DR2LUM (du Toit et al. 1984), the former in Cape Coloureds and the latter in the Xhosa.

A striking feature of the **genetic distances** (Table 5), calculated by the method of Cavalli-Sforza and Bodmer (1971), was the extreme dissimilarity (0.024) between the San and the Xhosa, the other African group studied. This was in spite of shared "African" genes and of the more recent gene flow between the San and Xhosa, predominantly from the former to the latter. The genetic distance measurement between the Cape Coloureds and the San was high (0.029) confirming that gene flow has been minimal between these two groups. The low values obtained between the Xhosa and the Cape Coloureds (0.012) as well as the Cape Coloureds and SA Caucasoids (0.010) suggested a close relationship between these respective populations. The genetic distances between the Xhosa and the SA Caucasoids, and the Caucasoids studied during the Ninth Histocompatibility Workshop, as shown in Table 5, were 0.033 and 0.034 respectively, both values considerably lower than that between the San and Caucasoids (0.050). This may indicate closer genetic links between the Caucasoids and Negroes, and is also in keeping with the view that the San must have evolved for a considerable time in relative isolation (Nurse et al. 1985).

Table 5. Genetic distances were calculated from the gene frequencies for the total number of antigens at the HLA-A, B,C and DR loci for the Xhosa, Cape Coloureds (Cape Col), S A Caucasoids (SA Cauc), San and Caucasoids from the Ninth Histocompatibility Workshop[1] (9WS Cauc).

	Xhosa	Cape Col	San	SA Cauc	9WS Cauc
Xhosa	0				
Cape Col	.012	0			
San	.024	.029	0		
SA Cauc	.033	.010	.047	0	
9WS Cauc	.034	.011	.050	.002	0

1 Baur et al. (1984)

HLA and Disputed Parentage

In an analysis of 3662 cases of disputed parentage, the HLA system alone excluded 98.08 percent of the SA Caucasoid, 95.68 percent of the Cape Coloured and 93.04 percent of the Xhosa non-fathers. In all three population groups, a significantly larger number of cases were excluded as a result of the B locus alone compared to the A locus. Between 60 and 70 percent of the HLA exclusions were based on both HLA-A and B locus alleles being different in the alleged father.

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other hand, shared haplotypes such as HLA-A1,B8 and A3,B7 with the SA Caucasoids and A30,Bw42 with the Xhosa. Two HLA-B,DR haplotyes, B7,DR2 and B8,DR3 were in strong positive linkage disequilibrium in the Xhosa, Cape Coloureds and the SA Caucasoids. The four most common HLA-B,DR haplotypes in the San, however, were different from those seen in the other three populations studied.

Table 4. Haplotype frequencies (HF) and linkage disequilibrium (ˆ) of significant HLA-A,B and B,DR associations (p<0.001), in SA Caucasoids (SA Cauc), Cape Coloureds (Cape Col), Xhosa and San.

	Haplotype				Haplotype			
	A	B	Δ	HF	B	DR	Δ	HF
SA	1	8	.057	.066	8	3	.056	.059
Cauc	3	7	.048	.071	7	2	.047	.067
	2	44	.018	.058	35	1	.029	.038
					62	4	.024	.035
Cape	30	42	.016	.019	8	3	.025	.028
Col	1	8	.017	.021	7	2	.023	.042
	3	7	.010	.019	57	7	.021	.026
					42	3	.017	.018
Xhosa	30	42	.039	.058	42	3	.051	.076
	28	70	.034	.056	7	2	.043	.052
	2	45	.023	.028	8	3	.023	.035
					45	1	.018	.017
San	43	7	.048	.054	7	6	.046	.074
	23	58	.047	.072	8	4	.038	.063
	2	8	.041	.051	70	4	.034	.068
	24	58	.030	.034	58	5	.027	.046

Whereas HLA antigens in Caucasoids appear to be well defined, **new variants** are still being discovered in the Negroid and Mongoloid populations. One of the classic examples of an HLA-A variant apparently confined to Southern Africa is HLA-Aw43, originally defined in the San (du Toit et al. 1984). A split of Aw43, recently detected in a small group of San, was also seen in the Cape Coloureds. The antigen Th is another example of an HLA-A locus antigen virtually confined to the Negroid populations. Among the HLA-B alleles, the B15 complex still has to be unravelled. We have described the B15 variant B15Kemp (Campbell et al. 1983a) in Cape Coloureds and recent studies in our laboratory suggest further splits of B15 in the Xhosa. A split of the Bw22 complex, 621-CT, was defined in the Xhosa (Campbell et al 1983b). During the Ninth International Histocompatibility Workshop, the division of Cw3 into Cw3.1 and Cw3.2 suggested by Chandanayingyong et al. (1981) was confirmed in our laboratory (Campbell and du Toit 1983). In the Xhosa only the Cw3.2 variant was present whereas in the SA Caucasoids Cw3.1 was most frequent. An additional complication in the definition of Cw3 was the segregation of Cw1 and Cw3.2 on the same haplotype in association with Bw46. Two HLA-DR2 splits have been observed in our

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Table 2. The Typical "Southern African" pattern for the HLA-A,B, C and DR loci.

	San n=289	Xhosa n=1027
	af	af
Common		
A28	.190	.276
A30	.329	.331
A23	.225	.154
Aw43	.201	.058
Th	.035	.086
Bw58	.498	.299
Bw70	.260	.291
Cw2	.273	.234
Cw6	.547	.391
	n=195	n=322
DRw6	.441	.329
Rare	n=289	n=1027
A1	.052	.068
A11	0	.003
A25	0	.001
B27	0	.003
Bw55	0	0
Bw56	0	0
Bw60	0	0
Bw61	0	.003
Cw1	0	.003
Cw5	0	.009
	n=195	n=322
DRw8	.015	.012

Table 3. Some HLA antigen frequencies which distinguish the San from the Xhosa.

	San n=289	Xhosa n=1027
	af	af
Increased		
A3	.266	.135
Aw43	.201	.058
B8	.225	.120
Bw41	.170	.031
Cw4	.291	.194
Cw6	.547	.391
	n=195	n=322
DR1	.287	.084
DR4	.441	.134
DRw9	.154	.016
Decreased	n=289	n=1027
A26	.004	.123
Bw42	0	.213
Cw3	.067	.133
	n=195	n=322
DR2	.072	.217
DR3	.108	.419
DR5	.133	.319

the Negroes, accounting for the differences in HLA frequencies that exist between them. The differences in these two populations that were originally closely related, may be due to genetic drift and the selective advantage of particular HLA alleles. Some of the principal HLA features distinguishing the San from the Xhosa were that the classical SA Negro antigen Bw42 was absent in the San while Aw43, B8, Bw41, Cw6 and DR4 were more frequent in this group (Table 3).

Some of the most common **HLA-A,B and B,DR haplotypes** are shown in Table 4. The HLA-A,B and B,DR haplotypes that were in strong positive linkage disequilibrium in the SA Caucasoids were the same as in the European Caucasoids (Baur et al. 1984). Different HLA-A,B haplotypes were seen in positive linkage disequilibrium in the Xhosa, San and SA Caucasoids. The Cape Coloureds, on the

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Table 1. Examples of significantly different HLA-A,B,C and DR gene frequencies in Cape Coloureds,Xhosa and San compared with SA Caucasoids.

	SA Caucasoids	Cape Coloureds	Xhosa	San
Increased	n=1059	n=3716	n=1027	n=289
A23	.021	.059	.080	.120
A28	.050	.082	.140	.100
A30	.026	.096	.182	.181
Aw34	.006	.026	.035	0[a]
Aw43	.002	.028	.030	.106
Th	0	.019	.044	.018
Bw42	.002	.032	.113	0[a]
B45	.010	.027	.050	.002[a]
Bw57	.031	.043	.054	.021[a]
Bw58	.011	.086	.163	.292
Bw70	.012	.081	.158	.140
Cw2	.055	.087	.125	.148
Cw6	.098	.171	.219	.327
	n=365	n=549	n=322	n=195
DR3	.096	.110[a]	.238	.054[a]
DRw6	.082	.109[a]	.181	.231
Decreased	n=1059	n=3716	n=1027	n=289
A1	.142	.086	.035	.026
A2	.269	.171	.130	.136
A3	.158	.077	.070	.144[a]
A11	.060	.065[a]	.002	0
A31	.023	.011	.002	0
B27	.040	.023	.002	0
B35	.083	.059	.021	0
B44	.150	.088	.065	.046
Bw60	.050	.023	0	0
Bw61	.012	.024[a]	.002	0
Bw62	.081	.045	.005	.028
Cw1	.032	.017	.002	0
Cw3	.140	.082	.067	.032
Cw5	.075	.019	.004	0
	n=365	n=549	n=322	n=195
DR1	.106	.057	.043	.149[a]
DR4	.152	.113	.069	.241[a]
DR7	.132	.135[a]	.074	.100
DRw8	.032	.027[a]	.006	.008

a Exception to the observed trend.

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linguistic, blood group and serological differences. However, it is virtually certain that the San share a common ancestry with the Negroes but have been separated for many centuries. Judging by the occurrence of their rock art, together with archeological and skeletal evidence, the San were spread over most of Southern Africa and are thought to have been the earliest known inhabitants. There are estimated to be 55,000 San still surviving, with about 15,000 in Namibia. They have had little contact with Caucasoids, and until recently lived as hunter-gatherers, like all human beings throughout the Pleistocene Age. The Negro influence has been limited mainly to a patron-client relationship.

RESULTS AND DISCUSSION

All the well recognized **HLA-A** locus antigens tested were present in SA Caucasoids, Cape Coloureds and SA Negroes, although striking variations in frequencies were seen (Table 1). The only HLA-A antigen not present in SA Caucasoids was the negroid' antigen Th. In the San, HLA-A11, A31, Aw34 and A25 were absent.

The classic Southern African Negro **HLA-B** antigens, Bw70 and Bw42 had their highest frequencies in the Xhosa, while Bw58 was highest in the San (Table 1). Although B8 has often been called a Caucasoid antigen (gf= 0.088), it was the most common in the San (gf=0.120) and also reached a relatively high frequency in the SA Negroes (gf=0.062). HLA-B27 was extremely rare in the Xhosa, present in only 3 of 1027 individuals, and absent in the San (Table 1).

Table 1 also shows the significantly different **HLA-C** gene frequencies in the different population groups. A feature in the Southern African Negroes and the San was the high frequency of Cw2 and Cw6 when compared to the SA Caucasoids and Cape Coloureds ($p<0.001$). HLA-Cw1 was rare in the Xhosa (gf=0.002) and absent in the San.

The commonest **HLA-DR** antigen in the SA Negroes was DR3 (gf=0.238) (Table 1), which occurs far more frequently than reported for all the race groups studied during the Eighth and Ninth International Histocompatibility Workshops (Baur and Danilovs 1980, Baur et al. 1984). The San have the highest frequency of DR4 (gf=0.241) which has a relatively low frequency in the Xhosa (gf=0.069).

When the HLA-A,B,C, and DR frequencies of the San and the Xhosa were compared, a typical **"Southern African"** pattern emerged (Table 2). This is best exemplified by the high frequencies of A30, Bw58, Cw6 and DRw6 and the virtual absence of A11, B27, Cw1 and DRw8. Most of the so-called classic Negro antigens reach their highest frequencies in the San, thus supporting the suggestion (Nurse et al. 1983; Mourant 1985) that the San share a common ancestry with, and may be even more "African" than the Negroes. On the other hand the San must have evolved for a considerable time in relative isolation without much contact with

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POPULATIONS STUDIED

a) South African Caucasoids

The SA Caucasoids, numbering 4 576 690 (1985 Census) originated from a relatively small genetic pool. Their ancestors were mainly the Dutch, who were the first settlers in 1652, with a substantial contribution from the German and French until 1700. The British immigrants arrived mostly in the 19th century. It has also been shown that SA Caucasoids have a further admixture of approximately seven percent Southern African and Asian genes (Botha et al. 1975).

b) Cape Coloureds

The Cape Coloureds, who now number over 2 825 094 (1985 Census), form an anthropologically distinct population group whose relatively recent origin is the result of early gene mixing at the Cape. They possess Western European, Southern African and South-East Asian genes, but in significantly different proportions from those seen in the SA Caucasoids. The indigenous Southern African component was derived mainly from the Khoikhoi (Hottentot) and to a lesser extent, from the San (Bushmen), as well as the Negro slaves who originated from Madagascar, Portuguese East Africa and the Gold Coast. Most of the South-East Asian genetic contribution came from the islands of the Dutch East Indies, Ceylon, and the shores and countries adjoining old India. The Caucasoid contribution was mainly from Western European settlers. The Cape Coloureds are therefore a classic example of a hybrid race of recent origin.

c) South African Negroes (Xhosa)

The Bantu-speaking Negroes of Southern Africa are divided into tribes. The two largest are the Xhosa and Zulu, each consisting of approximately 6 million, and they account for more than 50 percent of the Negro people of South Africa. From our own HLA data the Xhosa were shown to be representative of the majority of the other Southern African Negro tribes. The Xhosa belong to the Cape Nguni linguistic division of South-Eastern Bantu, and they have occupied the Transkei area in the south and south-east of the country for hundreds of years. There is evidence that Bantu-speaking Negroes crossed the Zambezi and journeyed south in a number of waves, between 500 and 1500 A.D. (Dart 1937). In 1652, when the first Dutch arrived at the Cape, they were still confined to the Transkei. Of all the Southern African Negroes the Xhosa are presumed to have had the greatest contact with the Khoikhoi and San.

d) San (Bushmen)

The present day San are descended from people of the later Stone Age living in Southern Africa as long ago as 10,000 years. They can be distinguished from the Southern African Negroes because of their shorter stature, more slender build, lighter skin colour, flatter faces and frequent steatopygia. There are also cultural,

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HLA POLYMORPHISMS IN SOUTHERN AFRICAN POPULATIONS

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INTRODUCTION

HLA is a powerful tool, as it is the most polymorphic genetic system known in man, determined by heredity in a known way, unchangeable during the life of an individual and observable by relatively simple blood tests. The knowledge of HLA polymorphisms may be applied to the study of the relationship between populations. The proportions of any particular set of antigens and those of the genes on which they depend, tend to remain constant from generation to generation, and any changes due to mutation, natural selection, and genetic drift are likely to be very slow. However, populations separated for many generations, such as the South African (SA) Negroes, the Caucasoids and the San, show variations in the frequencies of their HLA alleles and haplotypes.

The three major populations of the world are the Negroids, Mongoloids and Caucasoids. In this study the Negroids are represented by the Southern African Negroes (Xhosa) and the San. The SA Caucasoids belong to the Caucasoid group and the Cape Coloureds are a hybrid population with both Negroid and Caucasoid characteristics as well as some minor Mongoloid features.

The tremendous polymorphism of the HLA system and the low frequencies of the phenotypes make it extremely useful in solving problems of disputed parentage. It is, however, imperative that reliable gene frequencies exist for the population of the individual concerned. It is equally important that the investigator is familiar with the problems encountered in defining antigens in the population group to which a particular individual in a case of disputed parentage belongs.

The HLA-A,B,C and DR phenotypes of SA Caucasoids, Cape Coloureds, Xhosa and San were determined, and a comparison was drawn between their gene and haplotype frequencies. We also looked at the definition of some of the splits of the HLA antigens in these populations.

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Alloantigens on leukocytes and platelets: biochemistry

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Alloantigens of leukocytes and platelets are coded by genes located in the class I and II regions of the HLA complex. The class I region has at least 17 genes of which the HLA-A,B and C loci code for a glycosylated transmembrane polypeptide of 43kDa (kilo Daltons) which is associated non-covalently with β_2-microglobulin a non-glycosylated, non-polymorphic 12kDa polypeptide. The 43kDa polypeptide comprises three domains (α_1, α_2 and α_3) on the cell surface. Amino acid changes clustered in the α_1 and α_2 domains mediate class I antigen polymorphism. The α_3 domain and β_2-microglobulin are structurally homologous to an immunoglobulin constant region. The class II antigens are composed of two different, noncovalently associated, glycosylated transmembrane polypeptides (α and β) of 33 and 28kDa. Their respective genes are arranged into three subregions (DP, DQ and DR) which code for at least four groups of class II antigens. The β genes of all three subregions are polymorphic. However, whereas the DRα genes are non-polymorphic, the α genes of the DP and especially DQ subregions are polymorphic. The α and β polypeptides each comprises two domains on the cell surface, of which the two closest to the cell surface membrane are structurally homologous to an immunoglobulin region and the two most exposed domains mediate the serologically- and cellularly-detected polymorphisms.

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Serological detection of incompatible red cells is restricted to their presence in the circulation. Immunocytochemistry, however, additionally detects incompatible red cells or erythrocyte membrane remnants in the peripheral vascular region, in macrophages, and in necroses or hemorrhages. In this way, positive detection of an incompatible transfusion succeeds even after minimal transfusion doses or after a long survival time. The method is indispensable if blood samples are not available and/or if the question of a transfusion incident is to be investigated retrospectively on formalin-fixed autopsy material.

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The correlation between survival times and morphology

The morphological equivalents of shock were to be found in all cases. Within increasing survival time, the picture transformed from acute shock to the more complex pattern of protracted shock with its typical complications.These findings, however, ar not the subject of this study. The following will deal with the relation between the visualizability of IRC and survival time.

After survival times under 24 hours (n = 11), IRC were constantly present in the terminal vascular bed and were mostly also present in larger vessels. In all cases which could be evaluated, an intensive phagocytosis was already found in the liver, spleen and the adrenals. After very low transfusion volumes, IRC remnants occurred almost exclusively in macrophages.

After survival times of two to five days (n = 8), residues of IRC could be demonstrated exclusively in macrophages, but only after transfusion volumes of at least 450 ml. In four of these cases in which IRC were practically no longer demonstrable in normal tissues, they could be visualized without problems in necrotic tissue areas or in older hemorrhages. It is evident that the IRC had escaped phagocytosis in these unperfused tissues.

After survival times of eight to 17 days (n = 4), the results of attempts at immunocytochemical visualization of IRC were always negative.

survival time (days)	incompatible red cells in			
	large vessels	small vessels	macro-phages	hemmorrhages, necroses
0 - 1	+/-	+	+	n.p.
2 - 5	-	-	+/-	+
8 - 17	-	-	-	n.p.

Table: Demonstration of incompatible red cells (IRC), located intravascularly and in macrophages, depends on the survival period. Note the persistence of IRC in necroses and hemorrhages.

n.p. = not present in our material

CONCLUSIONS

The immunocytochemical findings make evident the two mechanisms by which IRC are eliminated from the circulating blood of the recipient. On the one hand, a redistribution of IRC takes place from the larger vessels into the capillary system even at short survival times. Since this process is evidently favored by the reduced perfusion in shock, the IRC are especially numerous in the capillaries of the "organs of shock", the lungs and the kidneys. On the other hand, in agreement with experimental findings (JANDL et al. 1957), massive phagocytosis of IRC, especially in the liver and the spleen, is already observed even after a very short time.

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RESULTS

IRC in large blood vessels

IRC, clearly identified by their staining behaviour, were present in large vessels as isolated cells or as circulating red cell agglutinations. These findings were constant in cases of massive transfusions and short survival times. However, therapeutic exchange transfusions as well as longer survival times resulted in complete disappearance of IRC from large vessels.

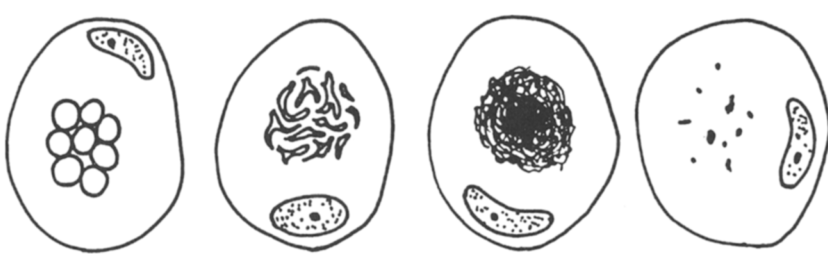
IRC in small blood vessels

With their occurrence in large vessels, IRC were simultaneously found in the blood capillaries. In some instances, lots of IRC were present in the terminal vessels whereas they had disappeared from the large vessels. The sinusoidal capillaries of the liver, spleen, the adrenal cortex and the bone marrow as well as the terminal vascular bed in the lungs and the kidneys proved to be sites of predilection for the occurrence of IRC, whereas the capillaries of the myocardium and the central nervous system showed strikingly few IRC.

IRC in mononuclear macrophages

Even after the shortest survival time of five to six hours, massive phagocytosis and intracellular degradation of IRC was to be observed in the stellate cells of the liver and in mononuclear macrophages of the spleen and the adrenal cortex. Four patterns which are to be interpreted as phases of a degradation process can be distinguished:

I closely packed intact IRC within macrophages;
II collapse of the IRC membranes;
III condensed, amorphous, globular masses;
IV granular IRC membrane residues.



Schematic: Representation of phagocytosis and intracellular degradation of incompatible erythrocytes by macrophages

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ABO incompatibility: Immunocytochemical findings in hemolytic transfusion reactions

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ABO incompatibility plays a major role in fatal transfusion reactions. However, a conclusive serological post mortem diagnosis may be a problem since the corpora delicti, namely the incompatible red cells (IRC), are quickly eliminated from the circulation by immunohemolysis and phagocytosis. On the other hand, IRC can be detected in the body tissues of the recipient using morphological methods. ISHIYAMA et al. (1977) and KEIL et al. (1983) reported encouraging results with the mixed cell agglutination reaction (MCAR). A better sensitivity and a higher resolution can be attained with more modern immunoenzymatic methods (PEDAL et al. 1986).

MATERIALS AND METHODS

Out of 23 autopsy cases after confirmed ABO-incompatible blood transfusions or erythrocyte transfusions, routinely formalin-fixed and paraffin-embedded samples of various tissues were available. The essential serological and clinical data were taken from documents of the Institutes of Forensic Medicine.

The transfusion volumes varied between 10 ml and 9.450 ml. Twenty recipients were blood group O; they had received A blood by mistake 15 times and B blood by mistake five times. Of three recipients with blood group B, two had received blood of group A and one had received blood of group A_1B. The survival times were between five hours and 17 days. The cause of death was hemolytic shock in all cases.

After deparaffination, and inhibition of endogenous peroxidase with 1 % H_2O_2-methanol and trypsin pretreatment, the antigens A and B were visualized with the standard PAP method in 4 µm sections. The incubation steps were:

1. monoclonal anti-A/anti-B Seraclone[R], Biotest, D-6050 Offenbach, cat. no. 801315/801340 (1:10)
2. rabbit antiserum to mouse IgM, Bionetics, distribution by Fresenius, D-6370 Oberursel/Ts., cat. no. 8403-09 (1:500)
3. swine antiserum to rabbit Ig (bridge antibody), Dakopatts, D-2000 Hamburg, cat. no. Z 196 (1:200)
4. PAP (rabbit), Dakopatts, D-2000 Hamburg, cat. no. Z 113 (1:100)

AEC was used as chromogen; counterstaining with hematoxylin.

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Das Antigen Lu^a fanden wir in 16 Fällen (39,0%), doch keiner gehörte der Blutgruppe O an, alle aber der Blutgruppe B oder AB. Lu^b fanden wir 22 Mal (53,6%), 3 Fälle zeigten kein positives Resultat (7,3%), Lu^aLu^a gab es 5 Mal (16,6%), Lu^aLu^b 11 Mal (36,7%), Lu^bLu^b auch 11 Mal (36,7%), Lu^{a-b-} 3 Mal (10%) (diese gehörten der Blutgruppe O, Rh D positiv an).

Tabelle:

System	Zahl d. positiven Resultate auf 30 Testierte	BLUTGRUPPE O (11)	A (11)	B (6)	AB (2)
Wr^a	-	-	-	-	-
Di^a	-	-	-	-	-
Jk^a	12	5	5	1	1
Jk^b	18	-	10	6	2
Xg^a	6	2	2	1	1
Js^a	1	-	-	-	1
Js^b	29	11	11	5	2
Lu^a	16	-	8	6	2
Lu^b	22	8	8	4	2
Bg	-	-	-	-	-
Kp^a	12	-	11	-	1
U	29	10	11	6	2
V	1	-	1	-	-
Tj^a	10	1	5	3	1
Bu^a	-	-	-	-	-

Obwohl es sich um ein kleineres Testgut handelt stellen wir fest, dass unsere Resultate bezüglich der Antigene Wr^a, Di^a, Js^a, Bg und Bu^a den schon bekannten Angaben aus der Literatur (als „selten" eingestufften) entsprechen (der Fall Js^a wird noch überprüft). Interessant ist die Beobachtung, dass Bu^a bei keinem Spender der Blutgruppe O gefunden wurde, jedoch bei allen der Blutgruppe B und AB. Die Verteilung der Blutgruppen Lu^a und Lu^b sowie Ik^a und Ik^b weicht von den in der Literatur angegebenen Werten nur etwas ab.

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Im Jahre 1974 wurde vorgeschlagen,das System Sm in Scianna-System umzunennen,die Faktoren Sm in Sc1 und Bu^a in Sc2,sodann die Alelle Sc^1 und Sc^2 (3).
In unserem Testgut fanden wir keinen einzigen Bu^a positiv.
Das Xg^a Merkmal rufte bei seiner Entdeckung eine echte Revolution aus,da alle bisherrigen Blutgruppen autosomal gesteuert werden,das Xg Merkmal ist aber auf dem X Chromosom lokalisiert.Das Xg^a hat bei Negern eine geringere Frequenz als bei Weissen.Vererbungsmässig kann von einem Xg(a+) Vater keine Xg(a-) Tochter entstammen,sowie von einer Xg(a-) Mutter kein Xg(a+) Sohn,soweit nur normale chromosomale Typen berücksichtigt werden,also kein Turner-Syndrom (X^o) oder Klinefeltersyndrom (XXY)(3).Einige bringen manchmal den Xg^a Faktor mit einigen Abnormalitäten bei Menschen in Zusammenhang,wie die rot-grüne Blindheit,Hämophilie A,Christmas›sche Krankheit oder der Muskeldystrophie des Typs Duchene.
Das Xg^a Antigen fanden wir sechs Mal,also in 20% und zwar bei zwei Frauen und vier Männern.
Der Faktor Kp^a (Penney) ist in Europa etwa in 2% zu finden, sonach wird der homozygote Typ mit 1:10.000 eingeschätzt. Durch die Zuordnung zum Kell-System wird Kp^a als K3 bezeichnet.Es besteht ein klarer Dosiseffekt.Wir fanden 12 Kp^a positive (40%);11 gehörten der Blutgruppe A an und einer (50%) der Blutgruppe AB.
Das U Antigen kommt häufiger vor.Vermutlich wird es durch ein Paar alleler Gene U und u vererbt.Es wird angenommen,das U ein nichtgeteiltes Ss ist,welches nicht separat mit Anti S und Anti s bestätigt wird.Es ist bekannt,dass M Erythrozyten Anti N binden insofern das N ein MU ist.
Das U Antigen fanden wir 29 Mal (96,6%).
Das V Antigen ist ein Rhesus Antigen,welches hauptsächlich in der schwarzen Bevölkerung zu finden ist,weniger bei den Weissen,dort wo das c,e Antigen in einer cis-Position zueinander stehen im Zusammenhang mit dem Chromosom cde und cDe (2).Natal hällt das V Antigen für eine Kombination von c und e^s (1).Wir fanden das V Antigen bei einer Blutspenderin der Blutgruppe A,Rh D positiv (3,3%).
Das Tj^a (Tumor Jay) Antigen ist ein Teil des P Antigens und zwar $P+P_1$ (2).Anti P_1 ist ein häufiges Kälteagglutinin,Anti $P+P_1$ hingegen ein sehr seltener Antikörper (1,2,3,4).
Hämolysine des Typs Tj^a (Anti P_1+Anti P_2) wurden öffters bei abortierenden Frauen gefunden (3).
Das Tj^a Antigen fanden wir 10 Mal (33,3%).
Das Js^a (Sutter) Antigen findet man in cca.20% der Negerblute während die Angehörigen anderen Rassen Js(a-) sind.Js^a wird als K6 bezeichnet (3).Das antithetische Js^b wurde in 99% der Neger und bei allen Testierten der weissen Rasse Js(b+) gefunden.Wir fanden Js^a bei einem Spender der Blutgruppe AB, Rh D negativ (wird überprüft).Der selbe ist auch Js(b+).Js^b fanden wir in 96,6%,nur eine A Rh D negative Frau war Js(b-).
Aus Interesse testierten wir auch Ik^a (Kidd) und Ik^b.Ik^a fanden wir 12 Mal (40%),Ik^b 18 Mal (60%).Kein Spender der Blutgruppe O gehörte dem Antigen Ik^b an.Ik^{a-b-}gab es 6 Mal (20%) sooft auch das Ik^aIk^a (20%) und Ik^aIk^b (20%),Ik^bIk^b aber 12 Mal (40%).Auffiel,dass alle Ik(a-b-) der Blutgruppe O angehörten.

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GLASER Edvard, Oddelek za transfuziologijo in imunohematologijo SPLOŠNA BOLNIŠNICA MARIBOR Jugoslawien

TESTIERUNGSRESULTATE EINIGER SELTENER BLUTGRUPPEN

Trotz der explosiven Entwicklung der Blutgruppenserologie seit der Entdeckung der ABO-Marker, hatten einige grössere Blutgruppenlaboratorien öffters auch heutzutage keine Möglichkeit aber auch kein Bedürfnis ausserordentlich seltene Blutgruppen zu testieren, obwohl solche Testierungen von grossem Wert sind-wie denn sonst käme man zu Erkentnissen einer ganzen Reihe ubiquitärer, familiärer, äusserst seltener oder an gewisse Rassen gebundene Blutgruppen oder Blutgruppensysteme. Es gibt aber auch noch eine ganze Reihe anderer Gründe, die für die serologische Bearbeitung der Blute und seltenen Antiseren sprechen, nicht zulezt auch Kompatibilitäts- und Paternitätsserologische u.A.
Natürlich spielen bei der Einführung solcher Teste auch andere Überlegungen eine Rolle neben den rein medizinischen Bedürfnissen, so rein labortechnische und finanzielle. Die Offenheit der Staatsgrenzen, die uneingeschränkten Reisemöglichkeiten der Menschen, sowie das so entstandene Völkergemisch gibt immer wieder Anlass zu neuen Forschungen.
In Jugoslawien gibt es noch keine Studie dieser Art. Auch wir haben eine turbulente Zeit mit vielen Menschenwanderungen hinter uns. So liegt es auf der Hand seltenen Blutgruppen in unserem Raum nachzugehen.
Wir verwendeten folgende Antisera (Ortho): Anti Di^a, anti Wr^a, Anti Xg^a, Anti Ik^a, Anti Ik^b, Anti Js^a, anti Js^b, Anti Lu^a, Anti Lu^b, Anti Bg, Anti Kp^a, Anti U, Anti V, Anti Tj^a und Anti Bu^a.
Wir testierten 30 Blutspender: 11 der Blutgruppe O (4 Rh D negativ, 7 Rh D positiv), 11 der Blutgruppe A (3 Rh D neg., 8 Rh D pos.), 6 der Blutgruppe B (2 Rh D neg., 4 Rh D pos.) und 2 der Blutgruppe AB (je einer Rh D neg. bzw. Rh D pos.).
Das Diego (Di^a) Antigen fehlt praktisch den Kaukasiern. Man findet es bei den amerikanischen Indianern, insbesonders bei den karibischen, aber auch bei Chinesen und Japanern (1,4) sowie indianischen Mischlingen. Das Di^a Antigen wurde anlässlich einer hämolitischen Erkrankung entdeckt. Nach Erwartung fanden wir unter unseren Probanden keinen einzigen Di^a positiv.
Das Wright (Wr^a) Antigen ist ein ebenso seltenes Antigen das seltener als 1:1000 zu finden ist (1,3,4). Wir fanden keinen einzigen.
Beim Testieren mit dem Anti Bg Serum fanden wir keinen positiv. Hier handelt es sich um das System Bennet-Goodspeed oder Sturgeon oder Donna (3), welches wahrscheinlich mit dem Leukozytenantigen HLA A7 in Verbindung steht, welches sich auch in kleinen Mengen auf den Erythrozyten mit spezifischen Antiseren beweisen lässt. Sicherere Resultate soll man mit dem Autoanalyser bekommen.
Auch das Bu^a Antigen ist ein sehr seltenes Antigen, welches eine Beziehung zum hochfrequentierten Sm Merkmal zeigt, denn Personen des Typs Sm(-) zeigen eine Doppeldosisreaktion mit Anti Bu^a, während Sm(+) Muster eine einfache Dosisreaktion zeigen oder Bu(a-) waren (3). So wären Sm und Bu antithetische Antigene.

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Table III depicts the comparative hemagglutinating activity of 19 floral extracts against human adult and cord red blood cells. The fruit pulp extract of Anthocephalus indicus showed positive specific hemagglutination reaction with cord red cells i.e. it gave a strong postive reaction with cord red cells and failed to react with human red cells. Three lectins Jacaranda mimosaefolia, Crotolaria mediciginea and Emblica officinalis showed negative specific reactions with cord red cells with respect to human red cells.

The results of the present study suggest that all the 10 mammalian red cells tested in the present study can be distinguished using either exclusive species specific hemagglutinins or a combination of hemagglutinins which react in an assortative manner. The fact that these lectins show species specific reactions with some species and not with the others tested does not rule out the possiblity that they will also not react with the red cells of species not tested. The present study also envisages that there are some differences as observed by hemagglutination by lectins on the red cell membrane between human adult and cord red cells. This informatin can be utilized for differentiation of human foetal blood from the adult blood in forensic serology. It goes without saying that a prior standardization of lectins specificities is essential before these could be of practical value in forensic examination of blood. A better understanding of the use of lectins in such investigations can be achieved through extensive screening studies carried out under conditions in which test materials are generally found at the site of crime.

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Table II. Lectins reacting in assortative manner.

Name of the Plant	Man	Monkey	Horse	Buffalo	Goat
Ptersperimum acerifolium	+	+	–	–	–
Citrulus festulosus	+	–	+	–	+
Citrus aurantifolia	+	–	–	+	–
Brassica oleracea	+	+	–	–	+
Antigonon leptopus	–	–	+	+	–

Table III. Serological activities of Lectins against Human Cord Red Cells.

Name of the Plant	Cord Red Cells	Adult Human Red Cells
Lycopersicum esculentum	1+	4+
Delinia indica	3+	1+
Jacaranda mimosaefolia	–	1+
Anthocephalus indicus	3+	–
Emblica officinalis	–	1+
Sechium edule	H	1+

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Table I. Serological Activity of Plant Extracts against Mammalian Red Cells

Name of the Plant	Man	Monkey	G.Pig	Rat	Rabbit	Sheep	Goat	Horse	Buffalo	Cow	Total
Lycoperscium esculentum	4+	4+	4+	4+	4+	3+	3+	4+	4+	4+	10
Solanum tuberosum	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	10
Coccinia indica	3+	3+	4+	3+	3+	3+	3+	3+	3+	3+	10
Brassica oleracea	ws+	1+	1+	-	1+	1+	1+	-	-	1+	7
Citrus aurantifolia	2+	H	2+	2+	H	2+	H	-	2+	2+	6
Citrulus festulosus	2+	-	2+	-	2+	2+	2+	2+	-	-	6
Antigonon leptopus	-	-	1+	1+	-	-	H	1+	1+	1+	5
Tamarindus indicum	-(1+)	-(1+)	-	-	H	-(1+)	-(1+)	-	-	-	4
Lagerstomia indica	2+	-(1+)	1+	1+	H	-	-	-	-	-	4
Achras sapota	2+	1+	1+	1+	-	-	-	-	-	-	4
Delinia indica	1+	H	1+	1+	-	-	-	-	-	-	3
Crotolaria medicaginia	ws+	ws+	1+	H	H	-	H	H	-	-	3
Adhotoda vesica	2+	-	2+	2+	-	-	-	-	-	-	3
Floscopa scans	-	-	-	-	-	-	-	2+	2+	2+	3
Tinospora cardifolia	-	-	-	1+	1+	-	-	-	-	-	2
Pterosperium acerifolium	4+	4+	-	-	-	-	-	-	-	-	2
Emblica officinalis	1+	H	-	1+	-	-	-	-	-	-	2
Rauwofia cauescens	-	-	-	2+	-	-	-	-	-	2+	2
Catharanthus rosens	-	1+	-	-	1+	-	-	-	-	-	2
Jacaranda mimosaefolia	1+	-	-	-	-	-	-	-	-	-	1
Arisaema specicosum	-	-	-	H	1+	H	-	-	-	-	1
Anthocephalus indicus	-	-	-	3+	-	-	-	-	-	-	1
Alocasia indicum	-	-	-	-	1+	-	-	-	-	-	1
Allium sativum	-	-	-	-	1+	-	-	-	-	-	1
Lantana indica	-	-	2+	-	-	-	-	-	-	-	1
Luffa acutangula	-	-	-	-	1+	-	-	-	-	-	1
Sechium edule	1+	-	-	-	-	-	-	-	-	-	1
Raphanus sativus	-	-	-	-	-	(1+)	-	-	-	-	1
Mimosa julfiflora	-	-	-	-	-	-	-	-	-	-	1
Total Number of Lectins Showing postive reaction	16	10	13	13	11	8	6	6	6	9	

\+ indicates haemagglutination in Saline;
(+) indicate activity in papain medium;
H indicates haemolysis in saline.

Strength of reactions
2+ - 4+ Strong reaction
w+ - 1+ weak reaction
ws+ Variable strength weak to strong.

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APPLICATION OF LECTINS IN FORENSIC SEROLOGY: A Simple Practical Method to Establish Source of Blood.

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INTRODUCTION

The knowledge that extracts of some plants agglutinate the erythrocytes of human and other animal species selectively, goes back to almost a century ago (Stillmark 1888). However it was not untill Bird (1954) suggested that lectins might serve to distinguish the erythrycytes of different animal species, that their utility in forensic examination of blood was recognized. Using extracts from four plant species (*Ricinus communis*, *Dolichos lab lab*, *Vicia faba*, and *Phaseolus lunatus*), Bird was also able to demonstrate that ten animal species could be clustered under only four groups. The pioneering efforts of Bird and other early workers provided useful leads in establishing the identity of blood, on the basis of differential agglutination reactions with lectins. A number of studies have been reported during the last three decades, reporting lectins which can be useful in distinguishing the blood of different animal species (Makela 1957; Sathe et. al.,1970; Arora and Sengupta 1976; Bhalla et al. 1979; Mastana 1983; Roy and Bhalla 1981).

The purpose of this paper is to present an overview of our investigations of serological activity of plant extracts with the red cells of a number of vertebrate species, and to demonstrate the use of plant agglutinins in establishing the source species of blood.

MATERIAL AND METHODS

In the present study 120 floral extracts of different plant parts such as fruits, stemtubers, leaves and roots have been investigated against red cell suspension of 10 mammalian species viz., man, monkey, rabbit, rat, gunieapig, horse, cow, buffalo, sheep and goat. Plant extracts were prepared in isotonic phosphate buffer saline,pH 7.4, by method described by Moore et. al.,(1972). Sodium azide (0.01) was added as preservative and the extracts were stored in deep freezer at -20 C. The agglutinating activity of the extracts was tested within one month of the prepration with a large number of red cell samples from each species. The redcells from different sources were obtained from animal houses and hospitals. The hemagglutinating assay was carried out with saline suspended red cells and papain treated red cells in phosphate buffer saline using standard serological techniques.

RESULTS AND DISCUSSION

In all 29 lectins showed non- specific or species specific or mono specific serological activity with the blood samples of 10 mammalian species (Table I). As evident from the results in Table I. the origin of blood of man, rabbit, guineapig, rat, cow and sheep can be achieved by employing species specific hemagglutinins. Red cells of monkey, buffallo, horse and goat can be differentiated by using a combination of lectins which react in an assortative manner, i.e. using one or two lectins to differentiate a species (Table II).

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Table 2. Mode channel of fluorescence of the child's red cells with anti-c

Phenotype	Anti -c	Blank	Difference
dce	84	43	41
dce	84	41	43
DCce	71	43	28
DCcE	65	39	26
DCe	38	40	0
Child	53	40	13

By comparing channels of fluorescence to the homozygous and heterozygous controls one can conclude that the child is heterozygous for the c antigen. This confirmed the titration studies. The data suggests that the c antigen in the child has a weaker strength than a normal heterozygous control. It is accepted that the C antigen produced by the R^z (*DCE*) gene, is less in quantity than that produced by R^1 (*DCe*). Commercial[2] anti-C typing reagents often contain mostly anti-Ce (anti-rh_i)[2]. Considering these two facts, the child was retested with additional anti-C reagents. The incubation times and the serum to cell ratio were increased and a weak C antigen was demonstrated on the child's red cells by using more sensitive techniques than those recommended by the anti-sera manufacturers.

DISCUSSION

Flow cytometry methods work well in determining antigen zygosity. However thorough knowledge of the instrument's capabilities is necessary to adjust it properly. For example, changes in the instrument's fluorescence gain can markedly enhance resolution between peaks. With the ability to change fluorescence gain, small differences among antigen strengths is more easily distinguished. Absolute differences of antigen strength can only be compared within a run since mode fluorescence channels will vary with the gain. Clear separation of the three cell populations is important in monitoring day to day fluctuation of the instrument's resolution and sensitivity. Careful instrument calibration is essential in order to obtain reproducible quantitative FACS measurement[3]. This is especially important when examining weak antigenic strength.

This method has been successfully applied to the determination of zygosity with anti-D and anti-E. However, at present, reliable results have not been obtained with anti-C and anti-e. The purity of anti-C antisera containing mostly anti-Ce presents problems not only with direct agglutination but also in FACS analysis. Optimal dilutions were never obtained for the C antigen in order to obtain clear separation of mixed cell populations of the C antigen. Thus the strength of the C antigen was not determined by FACS techniques in this case.

This study demonstrates the value of flow cytometry as a method for determining antigen zygosity and its application in the evaluation of the validity in a single indirect exclusion.

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The zygosity and antigen strength of each unknown red cell was determined by comparing the mode fluorescence channel with those of the control mixed cell population. Direct typing by conventional agglutination technique was then performed to determine the Rh phenotype.

RESULTS

The results of the phenotypic determination by agglutination and the corresponding fluorescence channels are displayed in Table 1.

Table 1. Mode channel of fluorescence with anti-c

Phenotype	Anti-c	PBS	Difference
dce	120	50	70
DCcEe	92	49	43
DCce	93	56	37
DCe	47	48	0
DC(c)(E)e	55	49	6

For each phenotype the mode channel of fluorescence using anti-c is compared with the mode of a blank control using PBS. The difference between the two values gives a semi-quantitative evaluation of the antigenic strength. It is apparent that results obtained by flow cytometry are consistent with the direct agglutination results. A homozygous c red cell demonstrates a difference of 70, when a heterozygous DCcEe has a difference of 43. Differences in channels among heterozygous phenotypes such as DCcEe (43) and DCce (37) may suggest varying degrees of antigen strength. The very weak c antigen of a red cell with the phenotype DC(c)(E)e, detected only by absorption-elution techniques, showed small changes in mode fluorescence channel (6) consistent with the weakness of the antigen. This phenomenum was consistently repeatable.

CASE STUDY

A paternity trio demonstrated a single indirect exclusion in the Rh system, with a residual paternity index of 178 (AF:DCEe M: DcEe; Ch: DcE) Titration studies with anti-c yielded the following results:

Child 1:2 (score 13); homozygous control 1:8 (score 25); and heterozygous control 1:2 (score 18).

Flow cytometry analysis with anti-c gave the following results.

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Flow Cytometry: An Alternative Quantitative Method to Determine Antigen Zygosity and Its Application to Paternity Testing

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Single indirect exclusions are a common problem in paternity testing. Approaches to resolve single indirect exclusions include: repeat testing with different reagents, testing for variant alleles, use of additional genetic markers and determining the zygosity by titration scores.[1] This study investigated the use of flow cytometry as an alternative technique for determining antigen zygosity as well as antigen strength.

MATERIALS AND METHODS

Samples of red blood cells were collected in acid citrate dextrose. 100 µl of a 0.25% suspension of red cells was incubated with 100 µl of a 1:2 dilution of commercial anti-c in U bottom microtiter plates and incubated for 30 minutes at 37°C. A blank control containing phosphate buffered saline (PBS pH 7.3) in place of antiserum was included. After washing twice with PBS, 100 µl of PBS was added to the dry cell button, followed by 100 µl of optimal dilution (1:20) of fluorescein conjugated Fab fragment of goat anti-human IgG heavy and light chains specific (FITC). This was incubated for 30 minutes at 22°C in the dark and washed twice with PBS. 100 µl PBS was added and each well content was transferred to FACS tubes, and analyzed on a Becton-Dickinson Fluorescence Activated Cell Sorter (FACS 420). The FACS made an analysis of 10,000 cells and printed a single histogram representing the number of cells versus channels of fluorescence. Each day a control consisting of a mixed population of cells of known phenotypes including a negative, a heterozygous and a homozygous cell was tested and used to set the FACS parameters (Fig 1).

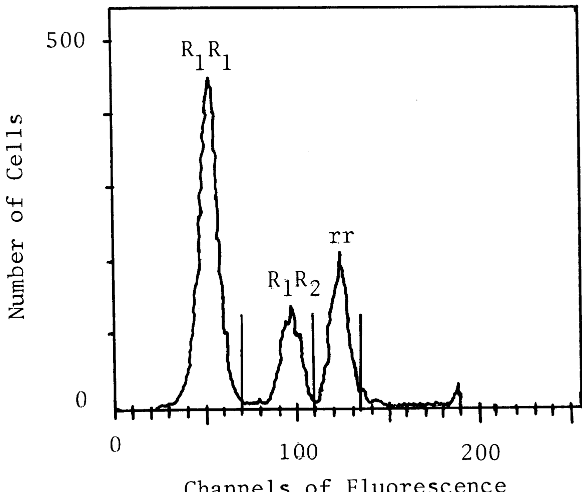

Figure 1 - Mixed cell population of cells negative, heterozygous and homozygous for the c antigen

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Partial structure of normal, EnaKT-specific GP A

```
        *     *     *
-A-H-E-V-S-E-I-S-V-R-T-V-Y-P-P-E-E-E-T-G-E-R-
 40                   50                   60
```

Proposed partial structure of Mi-VII-specific GP A

```
        *     *   * *   ?
-A-H-E-V-S-E-I-S-V-T-T-V-S-P-P-E-E-E-T-G-E-R-
 40                   50                   60
```

Proposed partial structure of Mi-VIII-specific GP A

```
        *     *   * *
-A-H-E-V-S-E-I-S-V-T-T-V-Y-P-P-E-E-E-T-G-E-R-
 40                   50                   60
```

Fig. 1. Proposed partial (res. 40-61) amino-acid sequences (one-letter code) and glycosylation sites (*) of Mi-VII- and MI-VIII-specific GP A molecules.

DISCUSSION

Previous data (Laird-Fryer et al. 1986) and the present results demonstrate that the Anek antigens of Mi-VII and Mi-VIII RBC are located within the res. approx. 40-57 of GP A. Although we have not performed any studies with anti-Lane and -Raddon, it is reasonable to assume that the Lane and Raddon antigens of these RBC are also located in this region of GP A. Laird-Fryer et al. (1986) showed that Mi-VII and EnaKT represent antithetical structures. The weak reactivity of Mi-VIII RBC with anti-En^{a}KT sera suggests that Mi-VIII and En^{a}KT are also encoded by alleles. Our finding that Mi-VII-specific GP A and Mi-VIII-specific GP A exhibit structural alterations within the region (res. 46-56) carrying the En^{a}KT antigen (Dahr et al. 1985) provides an explanation for these data at the molecular level.
Our results demonstrate that Mi-VII-specific and Mi-VIII-specific GP A exhibit an Arg - Thr (glycosylated) exchange at position 49. Mi-VII-specific GP A possesses an additional alteration (Tyr - Ser exchange at position 52). As judged from the genetic code, both sequence alterations can be attributed to two separate exchanges of single DNA bases in the gene for GP A. Therefore, it may be proposed that Mi-VIII represents the evolutionary link between Mi-VII and its normal counterpart.
Since anti-Anek, -Raddon and -Lane react more strongly with RBC from Mi-VIII heterozygotes than with those from Mi-VII heterozygotes (Dybkjaer et al. 1981), it may be concluded that they are predominantly directed against the glycosylated Thr-49. Laird-Fryer et al. (1986) showed that the Anek antigen of Mi-VI RBC is located on their altered GP B. It has been suggested therefore that the abnormal GP B molecules of Mi-III, Mi-IV and Mi-VI RBC were generated by gene conversion involving GP A and GP B as donor and recipient, respectively, during evolution. Structural studies on these RBC are necessary for a detailed understanding of the Mi-system.

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In a recent study (Dahr et al. 1985) we have shown that the high-frequency, trypsin-resistant, ficin-sensitive antigen En^aKT (previously denoted as En^aFS), detectable by alloantibodies from En(a-) individuals and autoantibodies from patients with autoimmune hemolytic anemia, is located within the res. approx. 46-56 of GP A. The finding (Laird-Fryer et al. 1986) that one individual (KT) whose RBC were found to contain a normal level of GP A had also made an alloanti-En^aKT provoked detailed studies. KT was shown to represent the first example of a Mi-VII homozygote. Her RBC were shown to lack the En^aKT antigen and RBC from Mi-VII heterozygotes gave a 'single dose reaction' with anti-En^aKT sera suggesting that Mi-VII and En^aKT represent alleles. The Anek antigen of KT's RBC was found to be located within the res. approx. 40-57 of GP A.

In more recent experiments we have determined the structural alteration of KT's GP A. Since the Mi-VII and Mi-VIII variants are closely related, we have also performed similar studies on RBC from two Mi-VIII heterozygotes (Dybkjaer et al. 1981; Vengelen-Tyler et al. 1985) (Dahr et al. 1987 and manuscript in preparation).

RESULTS

Just like RBC from Mi-VII heterozygotes, those from the two Mi-VIII heterozygotes yielded a 'single dose reaction' with four anti-En^aKT sera, thus suggesting that Mi-VIII and En^aKT also represent alleles. Two anti-Anek sera showed a slightly enhanced agglutination of trypsin- or chymotrypsin-treated Mi-VIII RBC and failed to react after ficin, V8 proteinase or sialidase treatment. These data suggest that the Anek antigen of Mi-VIII RBC is also located within the res. approx. 40-57 of GP A (data not shown).

Further evidence for this conclusion was obtained by hemagglutination inhibition assays: Anti-Anek sera were inhibited by the GP mixture, GP A and a tryptic peptide (T3) corresponding to the res. 40-61 of GP A from Mi-VII or Mi-VIII RBC. The mixture of GP B and GP C as well as the mixture of N-terminal tryptic peptides (T1, res. 1-39; T2, res. 1-31) from GP A had no detectable activity (data not shown).

The complete structure of T3 from the GP A of KT was deduced from amino-acid and carbohydrate analyses, manual DABTITC/PITC sequencing of T3 and secondary V8 proteinase fragments, carboxypeptidase Y digestion followed by amino-acid analyses and direct identification of the DABTH-derivatives of glycosylated Ser and Thr residues, as described in detail elsewere (Dahr et al. 1987). The T3 preparations from the two Mi-VIII heterozygotes were also sequenced in an automated gas-liquid solid phase sequenator (res. 40-60). The data demonstrate that the Mi-VII-specific and the MI-VIII-specific GP A molecules exhibit an Arg → Thr (glycosylated) exchange at position 49 (Fig. 1). In addition, Mi-VII-specific GP A was found to possess a Tyr → Ser exchange at position 52. It could not be elucidated whether Ser-52 is carbohydrate-free or glycosylated to a small extent.

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Biochemical Characterization of Class VII and VIII Cells within the Miltenberger System

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INTRODUCTION

The various antigens of the MNSs blood group system are located on two homologous, sialic acid-rich glycoproteins (SGPs) in human red blood cell (RBC) membranes: glycophorin A (GP A; MN SGP; α) and GP B (Ss SGP or δ) (for reviews see Dahr 1986; Lisowska 1987; Moulds & Dahr 1987). The 'MNSs locus', located on chromosome 4, appears to comprise two adjacent genes that encode the amino-acid sequences of these two molecules.

The Miltenberger (Mi-) subsystem of the MNSs system was originally (Cleghorn 1966) defined as a group of four (I-IV) rare RBC classes that share the Mi^a antigen, but differ with respect to other determinants (Vw, Mur, Hut, Hil). Some (V, VII, VIII) of the RBC classes chracterized more recently (Crossland et al. 1970; Giles et al. 1977; Dybkjaer et al. 1981) do not fit with the initial definition of the Mi-system in that they fail to react with anti-Mi^a. Mi-VII and Mi-VIII RBC are rather similar, since both RBC classes exhibit the Anek, Lane and Raddon antigens.

Previous studies (Dahr et al. 1984) revealed that Mi-I and Mi-II RBC exhibit a Thr → Met or a Thr → Lys exchange, respectively, at position 28 of GP A that prevents N-glycosylation of Asn-26. Mi-III, Mi-IV and MI-VI RBC were found to possess a GP B with an increased mol. mass (Dahr et al. 1978; Anstee et al. 1979). The Mi-V gene appears to encode a hybrid molecule of the Lepore-type comprising the residues (res.) 1- approx. 55 of GP A and the res. approx. 30-72 of GP B (Dahr et al. 1978; Anstee et al. 1979; Vengelen-Tyler et al. 1981), but no normal GP A or GP B.

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secreted by them.

The hemagglutination reaction between bacteria and erythrocytes are different from those between lectins and erythrocytes because the former is caused by bacterial elements and the latter is caused by soluble proteins. Many gram-negative bacteria such as enteric bacteria and Pseudomonas, and Corynebacterium have fimbriae (adhesive pili) on their surface as agglutinative organs. The hemagglutination reaction between fimbriae and erythrocytes are a non-reversible one because the reaction is inhibited by D-mannose. Little information is available on the study of soluble hemagglutinin. Hemagglutinin of B.pertussis is also fimbriae which was separated from the surface of the bacterium. However, the hemagglutinin of C. botulinus is secreted in culture medium is inhibited by D-galactose.

7. LANDSTENER'S PHENOMENA IN THE FIELD OF MICROORGANISM

In this study, blood group of the bacterium (Prokopoa nagaiensis) were examined. We performed absorption test by using the bacterium, and it was found that the bacterium had specific blood group antigen which is A-like antigen (Table 2, Fig. 1). Based on the concept of the "horror autotoxicus", that is, blood group A contains anti-B serum (Aß) and blood group B contains anti-A serum (Bα), these results obtained above are quite similar to "Landsteiner's phenomena" of blood group system. Therefore, we found the "Landsteiner's phenomena" in the field of microorganisms.

On the other hand, the bacterium infected with a bacteriophage could not produce the hemagglutinin. This phenomen is quite similar to situation of the human blood group system which is controled by regulator gene.

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Table 1. Hemagglutination of human erythrocytes by the Prokopoa nagaiensis hemagglutinin

	Blood group			
	A	B	O	AB
Hemagglutination titer	2	512	2	128

Protein concentration : 1 mg/ml

Table 2. Landsteiner's phenomena

	Bacterium[1]	Erythrocyte[2]
Surface antigen	A-like antigen	A-antigen
Hemagglutinin or Normal antibody	Anti-B	Anti-B (ß)

1: Prokopoa nagaiensis NAGAI 1981
2: Blood group A (Human)

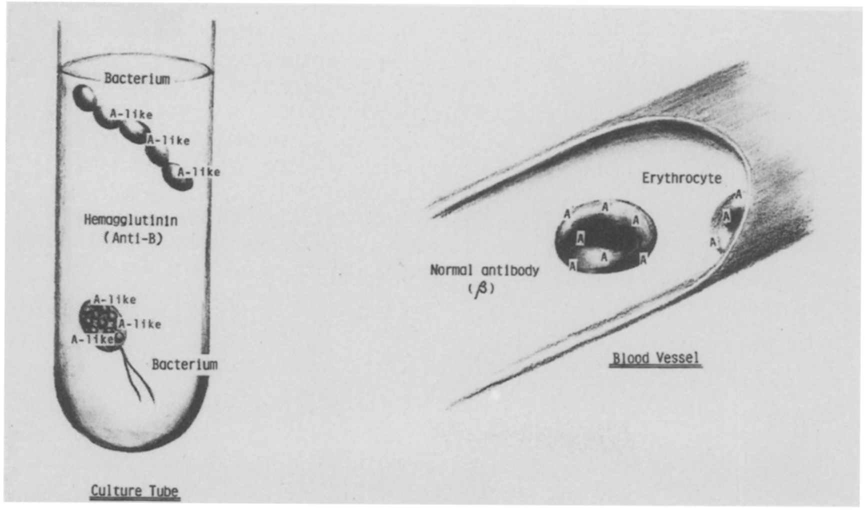

Fig. 1. Landsteiner's phenomena

but not by the derivatives with the lactone ring. ß-D-galactosides were more inhibitory than α-D-galactosides. L-rhamnose was the potent inhibitor among the monosaccharides tested, and its inhibitory effect was 10 times stronger than D-galactose. These results are the same as previous results which were obtained with purified samples.

6. HEMAGGLUTINATION IN OTHER BACTERIA:

We have many reports about blood group substances of bacteria; these reports are deeply concerned with blood group antigens. But there are few studies (Nagai and Prokop 1986) on interaction between blood group antigens of microorganisms and hemagglutinins

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the total activity found in the original culture filtrate.

2. MOLECULAR WEIGHT DETERMINATION:

The molecular weight was calculated to be 10,400 ± 500 from sedimentation equilibrium analysis. A similar value (10,300 ± 2,000) was also estimated from the mobility-molecular weight relationship on 5 % polyacrilamide gel in the presence method was 1.8 % which corresponds to 1 ml of hexose per 11,000 daltons. The result might suggest that the hemagglutinin contains a few moles of hexose per mole of the protein. Although the hemagglutinin actually contains some sugars, it is not certain at the moment whether they are bound covalently to the protein or not.

3. AMINO ACID AND CARBOHYDRATE ANALYSES:

Amino acid analyses of the hemagglutinin revealed that the contents of glycine, alanine and valine residues was very high, but no phenylalanine residue was present. Since the amount of carbohydrates detected in the protein is very low, we consider at present that the hemagglutinin may be not a glycoprotein.

4. BLOOD GROUP SPECIFICITY:

Optimam pH of the hemagglutinin reaction with human erythrocytes was pH 7-8. The hemagglutinin strongly agglutinated all the samples of human B and AB erythrocytes tested, and its anti-B activity was much stronger than the anti-A and anti-H activity (Table 1). The hemagglutinin has no specific activity for MN and P. The anti-B activity was confirmed by the fact that the purified hemagglutinin was inhibited by salivas from secretors with blood group B, but not inhibited by those from non-secretors.

Numerous hemagglutinins have been purified to homogeneity, but typical anti-B hemagglutinins have not been obtained from plants. In 1974, however, Hayes and Goldstein purified and characterized from Bandeiraea simplicifolia seeds a so-called anti-B lectin, the existence of which had been known through its anti-B activity (Mäkelä and Mäkelä 1956). The lectin is specific for α- galactpyranosyl residues, but agglutinates both human blood group A_1 and B erythrocytes. The hemagglutinating activity for A_1 cells is one-quarter of that for B cells.

In contrast, the purified Prokopoa nagaiensis agglutinin agglutinated human B erythrocytes 256 times as strongly as A or O erythrocytes. Thus, as far as serological activity is concerned, the Prokopoa nagaiensis agglutinin is more suitable to be called an anti-B hemagglutinin than the Bandeiraea simplicifolia lectin.

5. HAPTEN INHIBITION STUDIES:

The sugar specificity of purified hemagglutinin was examined by hemagglutination-inhibition tests. The hemagglutination was inhibited by D-galactose and its derivatives with the pyranose form,

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MATERIALS AND METHODS

1. CULTIVATION:

A medium was prepared according to the method of Nagai (1982). The growth of Prokopoa nagaiensis was continued for 7 days at 27°C. At the end of this period, the hemagglutinating activity reached a plateau of about 4 to 8 hemagglutination titer, and the culture broth was harvested by filtration with a piece of cloth.

2. ASSAYS:

Assay of hemagglutinating activity was performed with a micro-titer apparatus using a 2 % human A, B, O or AB erythrocyte suspension and a 0.15M NaCl solution as a diluent. Hemagglutination was conducted for 90 min. at room temperature. The activity was expressed as titer, the reciprocal of the highest two-fold dilution exhibiting positive hemagglutination.

For inhibition studies, purified hemagglutinin (titer, 4 units) was thoroughly mixed with varying concentrations of saccharides to be tested, and then a 2 % human B erythrocyte suspension was added to the mixture. The degree of inhibition was expressed as a final concentration of the maximum dilution at which the saccharide could inhibit hemagglutination.

3. PURIFICATION AND PHYSICOCHEMICAL ANALYSES

The procedures for the purification of the hemagglutinin from a new soil bacterium (Prokopoa nagaiensis) were previously discribed (Nagai and Prokop 1986). Physicochemical analyses such as disc electrophoresis, ultracentrifuge analysis, amino acid analysis and carbohydrate analysis for the hemagglutinin were previously discribed (Nagai and Prokop 1986).

RESULTS AND DISCUSSION

1. PURIFICATION OF THE HEMAGGLUTININ:

To 100 l of culture filtrate was added 500 ml of swollen cross-linked gum arabic gels, and this suspension was stirred 4 C overnight. The gel was collected by sedimentation and packed into a column. The column was washed successively with 0.15M NaCl, 1M NaCl and then with 0.2M D-galactose in 1M NaCl to remove contaminating proteins. The hemagglutinin was eluted by washing the column with 1M D-galactose in 1M NaCl. The active fractions were applied to a column of Sephadex G-15 to remove salts and sugars. The resulting hemagglutinin solution was lyophilized and stored at -80°C. The yield was about 270 mg, corresponding to 72 % of

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Studies on the Landsteiner's Model

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INTRODUCTION

Landsteiner (1901) published the report on the observation of the agglutination of normal human blood. He found that there were natural antibodies in serum which agglutinated the erythrocytes of other men. Landsteiner, whose remarkable research activity has been summarised in several monographs (Speiser 1961, 1962), initially discovered three blood groups, which were first called A, B and C, but were subsequently named A, B and O. Moreover, he found serum types against three blood groups mentioned above in serum. In 1902, Landsteiner's collaborators, Decastello and Sturli found fourth serum type which contained no "isoagglutinins". This was later named AB.

On the other hand, Schiff and Adelsberger (1924) first observed that there was a similarity of carbohydrate chain between human blood group A and Forssman antigen. After that, many researchers (Finland and Curnen 1938; Beeson and Goebel 1939; Iseki 1952; Springer 1956) found blood group A, B, H substances in gram-negative and gram-positive bacteria.

Race and Sanger (1975) indicated that the following studies were most contributory to ABO groups in past 50 years: (1) biochemical studies on A, B and H substances, (2) studies on Bombay type, (3) discovery of cis-AB, (4) discovery of specific hemagglutinin from plants and some invertebrates such as _Helix pomatia_. Thus, lectins play important roles in studies of blood groups. In 1965, Prokop and his collaborators (Prokop and Rackwitz 1965; Prokop, Rackwitz and Schlesinger 1965) reported a review of opinions and observations, they described the presence of group-like (antigen like) substances in plants and some invertebrates such as _Helix pomatia_.

We have many reports on the lectins extracted from plants and in vertebrates, but there are few studies (Nagai and Prokop 1986) on interaction between blood group antigens of microorganisms and hemagglutinins secreted by them. This report presents characterization and specificity of a hemagglutinin which was secreted from a new soil bacterium in Japan, _Prokopoa nagaiensis_ (Nagai 1982).

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pears to account for the known destruction of these antigens by reducing agents. The 93 KDa protein was found to form a disulfide complex with a 32 KDa component. It is tempting to speculate that the latter molecule is encoded by the Kx locus. The Kell antigens are firmly attached to the cytoskeleton (Dahr & Krüger 1983). Since Ge: -1,-2,-3 RBC are known to exhibit a depression of the Kell antigens, the 93 KDa might be attached to GP C rather than to a linking protein. Recently (Marsh et al. 1987), isolation of the 93 KDa component has been used for demonstrating that a novel low-frequency antigen (K23) belongs to the Kell system.

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LW SYSTEM

D-negative and D-positive RBC are known to contain different levels of 'D-like' antigens (LW^a, LW^b, LW^{ab}) that are absent from Rhnull and rare LW(a-b-) RBC and encoded by the LW locus. The LW^{ab} antigens were found to be located on a minor glycoprotein containing one or more Asn-linked carbohydrate chains (mol. mass about 43 KDa) that is firmly attached to the cytoskeleton (Moore 1983; Mallison et al. 1986; Anstee 1986). Intrachain disulfide bonds appear to be necessary for optimum antigen expression. This author assumes that the formation of complexes with the Rh proteins represents the prerequisite for incorporation of the LW molecule into the membrane.

DUFFY SYSTEM

The Fy^a and Fy^b antigens are located on a minor protein exhibiting a mol. mass of about 45 KDa as revealed by immunoprecipitation or -blotting (Moore et al. 1982; Moore 1983; Hadley et al. 1984; Lisowska et al. 1983; Dahr & Krüger 1983; Anstee 1986). Glycosidase digestions revealed that the molecule carries one or more Asn-linked carbohydrate chains. The Duffy-protein is not attached to the cytoskeleton, since it can be solubilized completely by a low Triton concentration. It contains cysteine, and therefore, may be separated from other components by affinity chromatography on Thiopropyl-Sepharose (Lisowska et al. 1983; Dahr & Krüger 1983). The possibility that the Fy5 antigen is represented by a complex involving Rh- and Duffy-molecules has already been mentioned above.

LUTHERAN SYSTEM

The protein(s) carrying the Lu^a and Lu^b antigens was found to exhibit properties similar to those of the Duffy-protein: It could be solubilized by a low Triton concentration and was bound by Thiopropyl-Sepahrose. However, the Fy and Lu antigens could be separated by ion exchange chromatography of Triton extracts (Dahr & Krüger 1983). Recent immunoblotting studies with a monoclonal anti-Lub showed that the molecule carries one or more Asn-linked carbohydrate chain(s) and exhibits a mol. mass of about 80 KDa (Anstee 1986; Parsons et al. 1987). It is not at all clear whether the Lu locus encodes the primary structure of the 80 KDa component. Udden et al. (1987) have presented evidence that Lu(a-b-) RBC of the In(Lu) type exhibit a carbohydrate alteration. Telen et al. (1984) have suggested that In(Lu) which represents a dominant suppressor gene, not linked to the Lu locus, regulates the expression of the 80 KDa component.

KELL SYSTEM

Redman et al. (1984) and Wallas et al. (1986) have shown that the K1 (K), K2 (k), K7 and K22 antigens are located on a minor glycoprotein possessing a mol. mass of about 93 KDa. This component exhibits at least one intra-chain disulfide bond that ap-

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Van Kim et al. 1987; Dahr et al. 1987c; Reid et al. 1987). Ge: -1, -2,-3 (Gerbich type) and Ge: -1,-2,3 RBC lack GP C and GP D, but contain a novel GP. Individuals exhibiting these phenotypes were found to exhibit a deletion (3 kilobases) within the GP C gene (Le Van Kim et al. 1987). The cytoplasmic portions of GP C and GP D are firmly attached to the linking protein band 4.1. Therefore, both GPs are firmly attached to the cytoskeleton, in contrast to GP A and GP B. GP C and GP D appear to represent the most important attachment sites for band 4.1. Therefore, Ge: -1,-2,-3 RBC (Leach type) are elliptocytic.

Rh SYSTEM

Earlier studies had provided evidence for the involvement of protein (cysteine) and phospholipids in the antigens of this system (for reviews see Issitt 1985; Salmon et al. 1984). The mol. mass of the protein(s) carrying the Rh antigens has been controversial for some time. There is now general agreement that the D, C, c, E and e antigens are located on one or more carbohydrate-free, hydrophobic minor protein(s) exhibiting a mol. mass of about 30 KDa (Moore et al. 1982; Moore 1983; Gahmberg 1983; Ridgwell et al. 1983; Krahmer & Prohaska 1985; Bloy et al. 1987). The molecule(s) could not be detected in Rhnull RBC (regulator and amorphic types) (Gahmberg 1983; Ridgwell et al. 1983). The Rh-proteins are only solubilized to a small extent by Triton extraction (Dahr & Krüger 1983; Gahmberg & Karhi 1984; Ridgwell et al. 1984). It is not yet known whether the molecules are directly attached to a linking protein such as band 4.1 or in an indirect manner via binding to a different integral protein such as GP C. Earlier studies (Moore 1983; Ridgwell et al. 1983; Krahmer & Prohaska 1985) provided some evidence that the D and C, c, E, or e antigens are located on separate molecules. Recent studies have clearly established the occurrence of at least two proteins and provided partial sequence information on the D-active component (Bloy, Blanchard, Dahr, Beyreuther, Cartron unpubl. data).

This author proposes the following hypotheses on the basis of the available serological, genetic and biochemical data: The C/c and E/e polymorphisms are represented by adjacent amino-acid exchanges on one protein. The D antigen is located on a homologous protein that is encoded by an adjacent gene. A deletion of this gene in dd individuals would provide an explanation for the high immunogenicity of the D antigen. The G antigen might represent (an) epitope(s) shared by the D- and C/c-, E/e-molecules. Since the Rh proteins in intact RBC are resistant towards proteinases, it may speculated that they are deeply embedded in the bi-layer. As judged from their mol. masses, they might span the layer severalfold. This author assumes that the Rh proteins exhibit the function to form complexes with certain different integral proteins within the intramembraneous particles (Dahr et al. 1987a) and thus, stabilize the attachment sites for the spectrin-actin meshwork. This hypothesis might provide an explanation for the hemolytic anemia of Rhnull individuals. Apart from alterations of the SsU antigens and a decreased level of GP B (Dahr et al. 1987a), already mentioned above, Rhnull RBC lack additional antigens (LW and Fy5) that are associated with the LW and Duffy systems.

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be necessary for optimum incorporation of GP B into membranes and for optimum expression of the high-frequency antigen U, located within the pos. approx. 34-40 of GP B (Dahr et al. 1987a; Dahr & Moulds 1987). In constrast to GP A, GP B lacks a long cytoplasmic portion. Its intramembraneous region is rather similar to that of GP A (Blanchard et al. 1987a).

Some time ago, it was shown that GP A and/or GP B are absent from (homozygotes), or decreased by about 50% (heterozygotes) in, RBC from individuals exhibiting rare silent alleles (En, u or Mk) at the 'MNSs locus'. Recent studies with cDNA probes have revealed that En(a-) or uu individuals exhibit a deletion of the GP A or GP B gene, respectively (Rahuel et al. 1987; Huang et al. 1987).

Certain rare MNSs alleles represent hybrid gene complexes. Lepore type genes encode hybrid GPs comprising an N-terminal portion from GP A (Mi-V and J.R.: res 1- about 55; English En: res. 1- about 26) and a C-terminal region from GP B (res. approx. 27-72), but no normal GP A and GP B. Typical anti-Lepore type alleles encode GP A, GP B and a hybrid GP comprising an N-terminal region from GP B and a C-terminal portion from GP A. The complete structures of the hybrids associated with the rare St^a and Dantu antigens were elucidated recently (Blanchard et al. 1987b; Dahr et al. 1987e). The St^a-hybrid exhibits the res. 1-28 from GP B and the res. 61-131 from GP A. The Dantu-hybrid possesses the res. 1-39 from s-specific GP B and the res. 72-131 from GP A. There are three varieties of the Dantu allele (Dahr et al. 1987f). The most frequent one, $Dantu^{NE}$, is an atypical anti-Lepore gene complex in that it does not encode GP B. The hybrid in $Dantu^{NE}$ RBC, produced in large quantity, is assumed to suppress the incorporation of GP A into the membrane in a cis and trans manner, via a competitive interaction with band 3 (Dahr et al. 1987b; Unger et al. 1987). As judged from data on the various hybrid GPs, the genes for GP A and GP B appear to be directly adjacent and exhibit the chromosomal alignment GP A-GP B.

GERBICH SYSTEM

The recognition that a rare variety (Leach) of Ge: -1,-2-3 RBC lacks the two minor SGPs GP C (D SGP or ß) and GP D (E SGP or γ) as well as the elucidation of the complete structure of GP C by protein and cDNA sequencing have paved the way for rapid analysis of the Ge system at the molecular level (for reviews see Dahr 1986; Reid 1986; Moulds & Dahr 1987). GP C (approx. 100,000 copies per single RBC) comprises 128 res., about 11 O-linked and one Asn-linked carbohydrate units. It exhibits a three-domain structure comparable to that of GP A. Knowledge of GP D occurring only in small quantity (about 20,000 copies per single RBC) is rather limited. The C-terminal portions of GP C and GP D appear to be identical. Both GPs share the Ge3 antigen that is located within the pos. approx. 40-55 of GP C. The Ge2 antigen occurs on the N-terminal portion of GP D, but not on GP C. It is not yet clear whether two separate genes at the Ge locus encode the peptide chains of GP C and GP D. The possibility that GP D represents an abridged version of GP C (res. approx. 20-128), generated by post-translational processing, is also being considered (Le

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MNSs SYSTEM

Several aspects of this system whose antigens are located on GP A (MN SGP or α) and GP B (Ss SGP or δ) have been covered by recent reviews (Dahr 1986; Lisowska 1987; Moulds & Dahr 1987; Reid 1986). The 'MNSs locus' is located on chromosome 4, as judged from previous conventional analyses and recent studies with a GP A-cDNA probe (Rahuel et al. 1987). It encodes the amino-acid sequences of two homologous proteins: GP A and GP B. The primary structure (131 residues, res.) and glycosylation sites of GP A were elucidated some time ago. Recent analyses of the cDNA sequence of GP A (Siebert & Fukuda 1986a) have paved the way for studies of genomic DNA that are about to be performed in three laboratories (Siebert & Fukuda 1986b; Rahuel et al. 1987; Huang et al. 1987).

GP A (apparent mol. mass about 35 KDa, about 900,000 copies per RBC) exhibits a hydrophilic, C-terminal, cytoplasmic domain (res. 96-131), a hydrophobic, intramembraneous region (res. 73-95) and an N-terminal, extracellular segment (res. 1-72) that carries about 15 O-glycosidic oligosaccharides (linked to Ser or Thr) and one Asn-linked carbohydrate unit at position (pos.) 26. Amino-acid polymorphisms at the 1st (Ser/Leu) and 5th (Gly/Glu) pos. represent the structural difference between the M and N antigens. Oligosaccharides at the 2nd, 3rd and/or 4th pos. are involved in the epitopes of many anti-M and -N sera. The rare M^c gene encodes an intermediate amino-acid sequence (Ser or Glu at the 1st or 5th pos., respectively) and thus, appears to represent the evolutionary link between the M and N genes. The rare M^g antigen was generated by a Thr → Asn exchange of N-specific GP A. This mutation prevents glycosylation at the 2nd, 3rd and 4th pos. of M^g-specific GP A. Other 'MN variants' (Tm, M_1, Can, Hu, Sext, N^a) appear to be caused by altered oligosaccharides (Dahr 1986; Moulds & Dahr 1987). Some rare MNSs alleles encode amino-acid exchanges at pos. 28 (Mi-I, Mi-II) or 49/52 (Mi-VII, Mi-VIII) that cause an altered glycosylation in these regions (Dahr et al. 1987d). GP A also carries several high-frequency antigens (En^aTS, res. about 26-42; En^aKT, res. 46-56; En^aFR and Wr^b, res. 62-72) (Dahr et al. 1986). GP A forms a specific complex with band 3 that is necessary for optimum incorporation of the molecule into the membrane and that might also exhibit a bearing on the Wr^a/Wr^b polymorphism, the complexity of which is not yet understood at the molecular level (Dahr et al. 1986, 1987b; Unger et al. 1987).

Structural analysis of GP B (72 res., about 11 O-linked oligosaccharides, apparent mol. mass about 24 KDa, about 300,000 copies per RBC) has been completed recently by studies of the protein (Blanchard et al. 1987a) and its cDNA (M. Fukuda pers. commun.). The N-terminal 26 res. of GP B are identical with those of blood group N-specific GP A, thus, providing an explanation for the occurrence of an additional N antigen (denoted as 'N') on GP B. The rare antigens He (exchanges at pos. 1, 4 and 5) and M^v (structure not yet known) are encoded by alleles of 'N'. A Met/Thr polymorphism at pos. 29 of GP B represents the structural difference between the S and s antigens. Studies on Rhnull RBC, which exhibit a decreased level of GP B, have provided evidence that GP B might form a specific complex with Rh-protein(s) that appears to

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Alloantigens of Proteins and Glycoproteins in Membranes of Human Red Blood Cells

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INTRODUCTION

Several blood group antigens, i.e. those belonging to the ABH, Lewis, P, I and Sid systems, consist of carbohydrate structures that occur on glycolipids or -proteins in human red blood cell (RBC) membranes. The corresponding gene loci appear to encode the primary structure of glycosyl-transferases (for reviews see Issitt 1985; Salmon et al. 1984). The MNSs system has served as a model for demonstrating that blood group loci may also encode the amino-acid sequences of structural proteins in RBC membranes. Evidence is now accumulating that a number of additional blood group antigens are specifically associated with various different minor (glyco)proteins. It is conceivable that the corresponding blood group loci encode the peptide sequences of these molecules.

RBC membranes contain basically two groups of proteins. The peripheral or extrinsic proteins are loosely attached to the cytoplasmic surface of the lipid bi-layer and can be released by changes of salt concentration or pH value. The major components, spectrin and actin (bands 1, 2 and 5), form a contractile meshwork that is pivotal for maintaining the biconcave shape of RBC. The integral or intrinsic proteins traverse the lipid bi-layer once or several-fold (in the case of the anion channel protein, band 3) with (a) hydrophobic domain(s). Their extracellular portions are usually glycosylated and may carry blood group antigens. The integral proteins form clusters within the bi-layer. There is evidence for specific complexes of different integral components within these clusters that appear to exhibit a bearing for certain antigens. The cytoplasmic portions of some integral components provide attachment sites for a special group of extrinsic molecules, denoted as linking proteins (bands 2.1 and 4.1). The linking proteins mediate reversible binding of spectrin/actin to integral proteins. The integral proteins can be solubilized by detergents such as Triton that purturb the interactions of their hydrophobic domains with lipids. The pellet obtained after extraction is referred to as cytoskeleton. It contains the peripheral proteins, lipids and those integral proteins that are not solubilized because of specific associations with cytoskeletal components. One group of integral proteins, the sialoglycoproteins (SGPs) or glycophorins (GPs), has been studied extensively, since the GPs can be prepared easily by extraction of membranes with organic solvents, due to their high carbohydrate content (up to 60% by weight).

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individuals; it is absent, however, in serum. The occurrence of the fucosyltransferases ("Secretor transferase" and "Lewis transferase") in mucous tissue parallels the Lewis blood group phenotype of the individual: in secretors where both enzymes - the α1-2 and α1-4 fucosyltransferase - are present, Le^b substance is synthesized, while in non-secretors, where the secretor gene dependent α1-2 fucosyltransferase is absent, only Le^a substance can be formed.

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this specificity is found in all sera except those of 'Bombay' (O_h) - individuals; in secretory tissue the enzyme is present only in secretors but is absent in non-secretors. In the classical model the secretor gene (Se) that determines the secretor status is considered a regulatory gene controlling the expression of H gene-specified fucosyl transferase in the secretory tissue. According to recent biochemical evidence, however, H and Se are both structural genes encoding two α1-2 fucosyl transferases with different characteristics: the secretor-transferase prefers to attach the α1-2 fucosyl residues to type 1 chains, and the H-transferase prefers type 2 chains.

The occurrence and distribution of the α1-2 fucosyl transferase show that the H gene-specified enzyme ("H transferase") is expressed in haematopoietic tissues and serum, and is absent in secretory tissues and secretory fluids, while the Se gene-specified enzyme ("secretor transferase"), on the other hand is expressed in secretory tissues such as gastric mucosa and submaxillary glands, and in secretory fluids. It is absent in haematopoietic tissues.

In non-secretors homozygous for the silent allele se, the "secretor-enzyme", the α1-2 fucosyl transferase, is absent. Since the A and B transferases are dependent on the H determinant as the precursor substrate, no ABH determinants can be formed in secretory tissue. The ABH antigens on the erythrocyte surface are unaffected, as they are synthesized by the action of the H-transferase.

The absence of the α1-2 fucosyltransferase in 'Bombay' serum is in full agreement with the earlier assumption that 'Bombay' individuals are homozygous for the silent allele h, which is not able to code for an enzymatically active α1-2 fucosyl transferase. In this case no A, B, or H antigens can be synthesized on the erythrocyte precursor cells. Since ABH active substances are also absent in body secretions, 'Bombay' individuals are probably ABH non-secretors.

The Lewis blood group is genetically independent of the ABH system; the appearance of the different Lewis characters, however, is closely connected to the secretor status of the individual: Lewis-positive individuals who are ABH secretors show Le^b specificity on red cells and in body secretions, while in non-secretors only Le^a activity is found.

Earlier investigations clearly show that the Lewis blood group substances of the erythrocytes are not synthesized in the red cells themselves but are absorbed from the plasma. Considering the fact that the Lewis phenotype is dependent on the secretor status, it can thus be concluded that the Lewis blood group substances are synthesized in a system influenced by the secretor gene.

The distribution of the α1-4 fucosyl transferase - the enzyme encoded by the Lewis gene - fully supports this assumption: the enzyme is found only in secretory tissue of Lewis-positive

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and urine.

The blood group phenotype expressed on the basis of genetic information is essentially dependent on the mechanism by which the blood group substances are formed. In contrast to proteins, which are synthesized in a continuing sequence translated from an mRNA, the oligosaccharide chains of the glycoconjugates are assembled stepwise by highly specific glycosyl transferases. The monosaccharide units are joined together according to a sequential mechanism, in which the product of each single step represents the acceptor substrate for the transfer step following it in the sequence, with sugar nucleotides (UDP-Gal, UDP-GalNAc, UDP-GlcNAc and GDP-Fuc) acting as monosaccharide donors.

It is generally accepted that one gene codes for one glycosyl transferase; and therefore a whole set of transferases, and thus genes, must be assumed for the biosynthesis of the whole carbohydrate chain. For the encoding of the enzymes responsible for the transfer of the blood group ABO(H) and Lewis determinant monosaccharide residues, three gene systems have been postulated - A/B/O, H/h and Le/le; the ability of the individual to secrete blood group ABH active substances in mucous tissues is controlled by the gene system Se/se.

According to this scheme the H gene codes for an α1-2 fucosyl transferase, the Lewis gene for an α1-4 fucosyl transferase, and the genes A and B for α1-3 N-acetylgalactosaminyl or α1-3 galactosyl transferases, respectively. The genes O, h, and le are considered silent alleles which do not encode enzymatically active glycosyl transferases. In cases where these alleles are present in double dose, i.e. on both chromosomes, the individual is not able to express A/B, H, or Lewis properties, resp.

The blood group gene dependent glycosyl transferases occur in serum and various tissues and have been investigated by the groups of Watkins (Lister Institute of Preventive Medicine, London), Ginsburg (National Institutes of Health, Bethesda, MD, USA), Schachter (University of Toronto, Ontario, Canada), and by our institute. The results of these investigations clearly show that the occurrence and the distribution of the enzymes in different organ systems is in close relationship to the respective genotype of the individual:

The A gene-dependent α1-3 N-acetylgalactosaminyl transferase is found in all individuals carrying the allele A, i.e. A and AB subjects; similarly, the B-enzyme - the α1-3 galactosyl transferase - occurs in all individuals carrying the allele B, i.e. B and AB subjects. In blood group O subjects with the silent allele O in double dose, no transferase is present to transform the H substance, and thus the occurrence of large amounts of the unchanged precursor substance is characteristic for blood group O individuals.

Human blood group H substance is produced from its precursor by the action of an α1-2 fucosyl transferase. A transferase with

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Fig. 1: The structures of the determinants of the blood group systems ABO(H) and Lewis.

Basic chain type 1: **Gal**ß1-3**GlcNAc**ß1-3**Gal**-

Basic chain type 2: **Gal**ß1-4**GlcNAc**ß1-3**Gal**-

H determinant: **Gal**ß1-3/4**GlcNAc**ß1-3**Gal**-
α1-2
Fuc

A determinant: **GalNAc**α1-3**Gal**ß1-3/4**GlcNAc**ß1-3**Gal**-
α1-2
Fuc

B determinant: **Gal**α1-3**Gal**ß1-3/4**GlcNAc**ß1-3**Gal**-
α1-2
Fuc

Le[a] determinant: **Gal**ß1-3**GlcNAc**ß1-3**Gal**-
α1-4
Fuc

Le[b] determinant: **Gal**ß1-3**GlcNAc**ß1-3**Gal**-
α1-2 α1-4
Fuc **Fuc**

type 1 chain ending with an α1-4 fucosyl group on the subterminal N-acetylglucosamine residue is responsible for the Le[a] character, while in the case of an additional blood group H determinant α1-2 fucose the specificity is changed to Le[b] (see Fig. 1). The serological determinant groups are attached to oligosaccaride units of various lengths which in turn are bound to sphingosine (→glycosphingolipids) or protein (→ glycoproteins); in the cell membranes glycosphingolipids as well as glycoproteins are present, while in secretions only blood group active glycoproteins occur. Group-specific oligosaccharides with analogous terminal structures have been isolated from milk

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Alloantigens on Red Blood Cells

Biochemistry of Red Blood Cell Antigens: Carbohydrate Antigens

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Blood groups normally defined by serological properties of erythrocytes are based on specific stereochemical configurations of the red cell membrane constituents. Since lipids in general are poor antigens, it must be expected that proteins and carbohydrate structures act as carriers of the antigenic determinants.

The antigens of the systems MNS and Rhesus are the most important within the protein-dependent blood groups. The determinants of the systems ABO(H), Lewis and P, the Ii-antigens, the so-called T-antigens and the characters Sid and Cad are based on oligosaccharide structures.

The first carbohydrate-dependent blood group systems to be investigated chemically were ABO(H) and Lewis. Thanks to the fact that the antigens of these systems are not confined to the red cell membrane but, rather, that great amounts of ABH and Lewis active glycoproteins are found in various body secretions (e.g. saliva, gastric juice and ovarian cyst fluid), it has been possible to isolate blood group active substances in quantities sufficient to determine the structures of the antigenic determinants.

According to the investigations of Morgan (Lister Institute of Preventive Medicine, London) and Kabat (Columbia University, New York), the carbohydrate chains carrying the ABO(H) and Lewis characteristics are built of four monosaccharides: D-galactose (Gal), L-fucose (Fuc), N-acetyl-D-galactosamine (GalNAc) and N-acetyl-D-glucosamine (GlcNAc). The basic structure of the A, B, and H determinants is represented by two types of carbohydrate chain endings - the terminus **Galß1-3GlcNAc-** ("type 1 chain") with no known serological specificity, and the terminus **Galß1-4GlcNAc-** ("type 2 chain") which is part of the I and i determinants and is responsible for the cross-reactivity of blood group substances with anti-type-14 pneumococcus sera. The chemical structures of the ABO(H) and Lewis serological determinants show that these blood groups are characterized by only one or two monosaccharide units:
An α1-2 fucose on the terminal galactose residue of a type 1 or type 2 chain is characteristic for blood group H activity. An H-specific structure with additional α1-3 N-acetylgalactosamine or α1-3 galactose shows A or B specificity, respectively. A

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